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(54) Title: METHOD FOR DIAGNOSING BLOOD CLOTTING DISORDERS

(57) Abstract

Assay methods for diagnosing blood clotting disorders are described. The assays use data bases for pooled normal plasma (PNP) and plasma from healthy volunteers, males and females ages 18 to 64 years. Charting on a comparative basis of patient plasma and PNP allows the results to be interpreted by reference to the data base. Simple, rapid, inexpensive and highly sensitive and specific assays devised for diagnosing blood clotting disorders are described.

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METHOD FOR DIAGNOSING
BLOOD CLOTTING DISORDERS

BACKGROUND OF THE INVENTION
(1) Field of the Invention

The present invention relates to an improved
method for diagnosing blood clotting disorders based upon
5   clotting times for a plasma from a patient. In particular,
the present invention relates to a method which uses a
combination of charts, pooled normal plasma (PNP) as a
control and data bases for normal and abnormal clotting in
the presence of various clotting or clot inhibiting agents
10   in diagnosing a specific clotting disorder.

(2) Prior Art

In the United States about 1.5 million people
per year have a heart attack. About half survive, and most
require thrombolytic (clot-dissolving) treatments like tPA
15   and streptokinase. About 500,000 people are hospitalized
each year in the U.S. because of pulmonary embolism (clot
in blood vessels in the lungs) or deep venous thrombosis
(clot in vessels of limbs, often in the legs). About
220,000 people per year have coronary by-pass surgery for
20   treatment of heart disease resulting from clots blocking
coronary blood vessels. 50,000 people die each year of
pulmonary embolism. 200,000 people die each year of
cerebrovascular disease. 50,000 persons in the U.S.
population have inherited clotting defects.

25   Diseases caused by blood clots are among the
most common life-threatening medical problems in the United
States. Another group of important diseases is caused by
failure of blood to clot (e.g. hemophilia); a lot of these
are inherited defects, but some are caused by other
30   conditions like cancer, Lupus, or even certain infections
that affect the liver and other tissues involved in the

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production of blood proteins. Clotting diseases are on the
increase because they are associated with life style
patterns in the western world (obesity, lack of exercise,
smoking), and with aging. People are living longer these
5   days and therefore more are entering the high risk age
groups. A lot more people are consequently put on oral
anticoagulants to counter this trend. New types of
treatment are now being developed that make it all the more
important to be able to reach decisions quickly on which
10   treatment to use and whether or not it is working properly.

        Accurate and rapid diagnosis of diseases caused
by clots or clotting defects is therefore a major everyday
problem for doctors and medical technologists.
Consequently, there is a significant segment of the
15   clinical diagnostic industry dedicated to providing
products to meet this demand.

        Laboratory diagnosis depends on tests done by
technicians to measure how long the patient's blood plasma
takes to clot, as compared to normal, and then to find out
20   exactly what is wrong when the plasma does not clot
properly. To do this, laboratories buy test kits and
diagnostic reagents that are designed to help identify the
clotting problem or defect. Some tests are simple; others
are complex, need expensive equipment and skilled
25   personnel, and take a lot of technician's time. Other
tests are so complicated or so costly in reagents that
routine laboratories in hospitals and clinics don't even do
them, so they send the blood sample away to a specialty
laboratory where the tests are performed by experts for a
30   fee.

        Several companies in the medical diagnostic
field sell coagulation diagnostic kits. However, these
kits are mostly based on technological principles developed
in the 1950's and the procedures are frequently seriously
35   flawed. Modernization in technique has come primarily in
the form of automation by computer-controlled instruments
(coagulometers) that provide increased accuracy and avoid

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human errors, and reduce technician time, but the test principles are still the same. These new instruments are expensive, particularly in their most automatic form. They handle multiple samples and have built-in micro-processors

5   that perform all the computations of results and comparisons to normal values. For some of the tests needed for monitoring clot dissolving treatments (e.g. urokinase, tPA), additional equipment is needed.

The trend in the industry toward automation has

10   come about due to the high cost of technical time, and the demand for more, faster and better diagnostic testing. As pharmaceutical companies develop totally new drugs the need for more tests, performed more frequently and giving more accurate information to the doctor, will increase

15   substantially. Clot-dissolving products are expected to be among the biggest growth areas in the medical therapy market over the next decade. For example, based on sale after FDA approval, tPA is the most successful new drug ever introduced in the United States. The diagnostic

20   industry is going to have to move in the direction of speedier tests, performed as close as possible to the patient and his or her physician. The needs for certain kinds of tests that monitor, for example, tPA infusion therapy or oral anticoagulant prophylaxis will increase.

25   The same is true for the "replacement" treatments that are now appearing for genetic and acquired clotting defects; these new therapies have only become possible because of genetic engineering technology. There are no satisfactory systematic diagnostic approaches available in

30   the marketplace to meet these needs at the moment. There is a need for such a systematic approach.

The prior art procedures employed in most cases are flawed; they are outdated and have not been optimized to meet the level of critical, quantitative diagnostic need

35   in today's clinical setting. Reagents of uncertain value or giving unreliable results are used on the basis of tradition rather than on the basis of rigorous scientific

evaluation. Consequently, it is not possible to reach a
correct and accurate diagnosis using many of them, and they
do not match up to expectations of the clinician for
therapeutic monitoring. The more complex tests available
5   today for monitoring need equipment and skills that are not
routinely available, and therefore they are only done in
reference lab settings; although the clinician needs these
kinds of test results, the procedures are not run often
enough to provide the best results. In some cases the
10   current diagnostic reagents are not stable and their shelf
lives are not well characterized for today's quantitative
tests. The variables that influence the test results, like
disease state and therapeutic history, have not been
examined sufficiently in most cases to permit these kits
15   and reagents to be used with confidence for quantitative
purposes. Quantitative test data is needed when powerful
therapies are being applied that can be disastrous in the
wrong situation.

The complications faced by the clinician in
20   dealing with these new surgical and medical interventions
becoming available for patients, coupled with the
complexity of the lab technology, have resulted in a
serious communication gap between the two. There is a need
for a comprehensive approach to differential diagnostic
25   logic in thrombotic and clotting diseases, in a modern form
that makes the system available and understandable to both
clinical and laboratory personnel.

OBJECTS

It is an object of the present invention to
30   provide tests which have characterized reagents, improved
configuration of the assays, quantitative reliability in a
wide variety of disease states, and proven
interrelationships of test results with clinical and
pathological data. Further it is an object to provide
35   tests which are relatively simple and economical. These
and other objects will become increasingly apparent by
reference to the following description and the drawings.

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IN THE DRAWINGS

Figure 1 is a Table showing the results of testing for prothrombin (P), activated partial prothrombin (APT) and thrombin (T) in identifying various blood coagulation deficiencies according to the present invention.

Figure 2 shows the various times corresponding to various blood coagulation deficiencies according to the present invention.

Figure 3 shows the mean of the clotting time of pooled normal plasma (PNP) and Factors V, VII and X genetically deficient plasma (less than 1% Factor activity) which is measured by the P assay.

Figure 4 shows the comparative clotting time by the APTT assay for zero percent Factor activity as indicated.

Figure 5 is a Table showing the clotting times of various assays and the diagnosis.

Figure 6 shows the normal range of the mean clotting time of 233 individuals, ages 18 to 64, using citrated human plasma samples measured by the P time assay. The mean is 11.4 and the median is 11.3. The range is 10.3 to 12.7. PNP (100 ul) was clotted with reconstituted 200 ul tissue thromboplastin/calcium chloride (TTP/CaCl$_2$ powder, Ortho Diagnostic Systems, Raritan, New Jersey) to give a clotting time of 11.6 ± 0.5 seconds with PNP.

Figure 7 shows the normal range of the mean clotting time of 221 individuals using citrated human plasma samples measured by the APT time assay. The mean is 26.6 and the median is 23.5.

Figure 8 is a chart characterizing the various factors in blood plasma.

Figure 9 is a table showing the various tests which are used to finally diagnose a thrombosis disease. The hemostasis assays of the present invention are shown in the context of this diagnosis.

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Figure 10 is a chart which is used for recording the assays for factors of the entrinsic pathway.

Figure 11 is a chart which is used for recording assays for the factors of the intrinsic pathway.

5

Figure 12 is a chart which is used for recording assays for inhibitors.

Figure 13 is a chart which is used for recording the assays of the factors of the contact phase of plasma activation.

10

Figure 14 is a chart which is used for recording the assays of the antithrombin III assays.

Figure 15 is a chart which is used for recording the assay for protein C.

Figure 16 is the chart used for recording the

15   assay for fibrinogen.

Figure 17 is a chart which is used for recording a bleeding workup.

Figure 18 is a chart which is used for recording a hypercoagulation workup.

20

Figure 19 is a graph showing fibrinogen concentration versus clotting time.

Figure 20 is a graph showing the results of a fibrinogen assay for various patients.

Figure 21 is a graph showing plasma fibrinogen

25   versus P time.

Figure 22 is a graph showing the results of testing for antithrombin III.

Figure 23 shows the linear regression clotting time of PNP containing progressive concentrations of bovine

30   lung heparin by the APT assay. The results are representative of those for heparin from other sources.

Figure 24 determines whether 40 percent factor activity was equivalent to 80 percent factor activity for Factor V. In the test, 50 ul single factor genetically

35   deficient plasma was mixed with 50 ul single donor plasma in the PT and APTT assays. From the bar charts it is seen that clotting times by the APTT and PT assays for 40

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percent plasma levels of Factors V, VII and VIII are very
close to the clotting times of 80 percent levels of the
clotting factors.

Figure 25 determines whether 40 percent factor
activity was equivalent to 80 percent factor activity for
Factor VII. In the test, 50 ul single factor genetically
deficient plasma was mixed with 50 ul single donor plasma
in the PT and APTT assays. From the bar charts it is seen
that clotting times by the APTT and PT assays for 40
percent plasma levels of Factors V, VII and VIII are very
close to the clotting times of 80 percent levels of the
clotting factors.

Figure 26 determines whether 40 percent factor
activity was equivalent to 80 percent factor activity for
Factor X. In the test 50 ul single factor genetically
deficient plasma was mixed with 50 ul single donor plasma
in the PT and APTT assays. From the bar charts it is seen
that clotting times by the APTT and PT assays for 40
percent plasma levels of Factors V, VII and VIII are very
close to the clotting times of 80 percent levels of the
clotting factors.

Figure 27 determines whether 40 percent factor
activity was equivalent to 80 percent factor activity for
Factor VIII. In the test, 50 ul single factor genetically
deficient plasma was mixed with 50 ul single donor plasma
in the PT and APTT assays. From Figures 24 to 27 it is
seen that clotting times by the APTT and PT assays for 40
percent plasma levels of Factors V, VII, VIII and X are
very close to the clotting times of 80 percent levels of
the clotting factors.

Figure 28 shows standard curves in factor
deficient plasma. It was proved that there is a lack of
scientific evidence for use of 20 percent plasma to buffer
ratio as equivalent to 100 percent activity. It was shown
that there was a loss of activity for FV and FX in the PT
and APTT assays at 35 percent plasma concentration. Twenty
percent activity is not equivalent to 100 percent for

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several factors. Yet, factor assays in all hospital
laboratory settings are performed on that incorrect
assumption.

5   Figure 29 shows standard curves for descending
ranges of Factors V, VIII, IX, X and XI by the APTT assay.
GENERAL DESCRIPTION

          The present invention relates to a method for
diagnosing blood clotting disorders in humans which
comprises: separately testing sets of a sample of plasma
10   separated from the blood of a patient and sets of a sample
of plasma from pooled normal plasma (PNP) from healthy
humans for clotting time (CT) by addition of predetermined
amounts of prothrombin (P) to a first set of the sample;
activated partial thromboplastin (APT) to a second set of
15   the sample and thrombin (T) to a third set of the sample,
charting the results for the patient and the PNP together
on a side-by-side basis for P, APT and T and comparing the
results with a data base showing normal ranges of CT based
upon the PNP for APT, T and P wherein the APT, T and P have
20   been separately prepared in solution to produce a
particular standardized clotting time with PNP which is
used in all of the testing of the patient plasma; testing
for hypercoagulation or bleeding based upon the P, APT and
T tests and charting the results; and providing a diagnosis
25   based upon the differences of CT based upon the tests.

          Further the present invention relates to a
method for diagnosing blood clotting disorders in humans
which comprises: separately testing a first set of samples
of plasma from a patient and pooled plasma from normal
30   healthy humans (PNP) for the time to coagulate by
prothrombin (PT), activated partial thromboplastin (APT)
and thrombin (TCT), charting the results together on a
side-by-side basis, and comparing the results from the
samples with a data base showing abnormal and normal ranges
35   of coagulation times (CT) for healthy humans, wherein the
APT, T and P have been prepared in solution to produce
particular standardized clotting time with PNP which is

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used in all of the testing of patient plasma; optionally
testing second sets of a sample of the plasma from a
patient and PNP by mixing the patient plasma suspected to
be genetically deficient in a blood factor selected from
5   the group consisting of Factors V, VII, VIII, IX, X, XI,
XII, Fl.F and HMWK with a volume factor deficient of a
genetic plasma in an amount between about 40 to 60 percent
by volume of GFPD to PNP and patient plasma for coagulation
by an appropriate one of PT, APT or both separately,
10   charting the results together on a side-by-side basis and
comparing the results with a second data base showing
normal ranges of CT based upon PN wherein the CT of GFPD is
corrected by the PNP and normal patient plasma; optionally
testing the samples of the plasma for anti-thrombin III by
15   determining CT for the patient and PNP, charting the
results together on a side-by-side basis and comparing the
results with an antithrombin III data base showing normal
ranges for PNP; optionally testing the samples of the
plasma for protein C by determining CT and charting the
20   results and comparing the results with a protein C data
base showing normal ranges of CT based upon the PNP;
optionally testing the samples of plasma for fibrinogen
from the patient plasma and PNP and determining CT for
coagulation by T at known dilutions of the plasma, charting
25   the results as CT on a side-by-side basis and comparing the
results with a fibrinogen data base showing normal ranges
of CT for the PNP; and providing a diagnosis based upon the
differences in coagulation times in the tests.

        In the present invention the PNP is used to
30   rigidly standardize the CT reagents P, AP and T and are
formulated to always produce the same CT with PNP. For T
the CT is preferably 8 to 9 seconds. This is 1.2 units per
100 ul of solution. For AP the CT is preferably 26.4
seconds $\pm$ 2 seconds. For P the CT is preferably 11.6
35   seconds $\pm$ 1 second. Generally the tests are conducted
in 200 ul of PNP. If this procedure is not followed, the
results of the assays will be variable from lot to lot of

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P, T, and APT and from day to day and the results in the manner of the present invention can not be achieved which relies upon producing the same CT with normal plasma.

It has been found that when PNP is mixed with a Factor deficient plasma, in a volume between about 40 and 60% of the volume of the Factor deficient plasma (GFDF) in a PT, APTT or TCT/TT assay that the PNP will correct the factor deficiency and if it is normal, the patient plasma will correct the factor deficiency of a factor deficient plasma. If there is a particular factor deficiency in the patient plasma it will not correct the GFDF thus showing that the patient plasma is also deficient in this factor. This mixing technique is a basic concept of the present invention. Histograms for FVII, FX and FV by the PT assay and FVIII by the APTT assay are shown in Figures 24 to 28.

Diseases associated with hemostatic disorders are broadly categorized into bleeding and thrombosis. Bleeding can be external or internal. Internal bleeding includes the types that manifest under the skin such as hematomas, bruises, purpura and petichiae. Thrombosis can be acute or chronic. The acute form of thrombosis if untreated, will result in severe uncontrollable bleeding. Chronic thrombosis can be caused by deranged protein coding or by environmental factors.

To investigate the pathogenesis of bleeding and thrombotic diseases a multitude of standardized procedures were developed. Some are complicated, with a high degree of sophistication and can be carried out only in research laboratories. Others are relatively simple and suitable for clinical testing.

Figures 1 to 8 show the diagnosis of various diseases and the CT for normal and abnormal patient plasma by the APT, PT and T tests. These tests are performed and charted as shown in Figures 10 to 18. The individual assays are for factor deficiency, inhibitors, contact phase antithrombin III, protein C, and fibrinogen as well as other standard assays as shown in the charts.

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SPECIFIC DESCRIPTION
Routine Assays

There are several well established screening·
procedures of a very general nature which are ordered
5   routinely on individuals who present for the first time
with a bleeding disorder.

(1) The Tourniquet Test (Rumple Leede Test).
This is a non-invasive, easy to perform procedure to
identify capillary fragility. It is performed by applying
10   a blood pressure cuff for 20 to 40 minutes and applying a
40 mm Hg pressure. In fact, it is the only available
testing procedure for the blood vessel component of the
hemostatic system. Blood vessel disease is the least
commonly encountered hemostatic abnormality. It is
15   frequently seen associated with viral infections, drug
induced vasculitis, and collagen diseases such as lupus
erythematosus and others. It is also frequently seen in
the elderly.

(2) Platelet Count. A platelet count of 50,000
20   per cubic milliliter of blood ensures adequate hemostasis
unless challenged by stressful conditions such as trauma,
surgery or childbirth. Platelet counts of 10,000 per cubic
milliliter of blood are dangerously low and may lead to
spontaneous hemorrhage. Thrombocytosis is a platelet count
25   above 500,000 per cubic milliliter. Thrombocytosis
predisposes to thrombosis in most cases. In
myeloproliferative disorders platelet counts may reach 1
million per cubic milliliter. In such conditions platelet
functions are defective and thrombocytosis is associated
30   with excessive bleeding rather than thrombosis.

(3) Bleeding Time Tests. The Duke and Ivy
time bleeding procedures are technically difficult to
perform. Adequate standardization is essential for the
interpretation of the results. In expert hands a normal
35   bleeding time of greater than 15 minutes is frankly
abnormal and indicates:

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Severe impairment of platelet functions of genetic or acquired origin.

Very low blood levels of von Willebrand Factor.

Afibrinogenemia and severe Factor V deficiency.

A bleeding time greater than 8 minutes and less than 15 minutes is more difficult to interpret. A moderately low plasma level of vWF is by far the most common underlying pathogenesis. Antiplatelet drugs, lupus-like inhibitors and Factor XI deficiency should be considered in the differential diagnosis.

(4) Prothrombin Time (PT). The PT is a screening test to identify coagulation abnormalities of the extrinsic and common pathways and fibrinogen. This test is performed by mixing tissue thromboplastin/calcium chloride solution (TTP/CaCl$_2$) with the plasma. Oral anticoagulant medication is the most frequent cause of prolonged PT. The only abnormality that causes a prolonged PT and no changes in the other screening tests is a Factor VII deficiency. The PT is sensitive to slight decreases in plasma levels of Factor V.

(5) Activated Partial Thrombo-Plastin Time (APTT). The APTT is a screening test to identify coagulation abnormalities of the contact phase and the intrinsic and common pathways of plasma activation. This test is performed by mixing activated partial thrombin reagent (APTT) with the plasma. The APTT is not sensitive to fluctuation in plasma fibrinogen levels. Of conditions that cause prolongation of the APTT, the most frequent is contamination of plasma with heparin. Other conditions are Factors VIII and IX deficiencies as well as lupus-like anticoagulants. A factor XII deficiency of less than 1% gives an APTT of 260 to 300 seconds, while deficiencies of Factor VIII or IX of less than 1% will give a more modest increase of 78 to 82 seconds in the APTT.

(6) Thrombin Clotting Time/Thrombin Time Assay (TCT/TT). TCT or TT is the same assay given two slightly different names. TCT/TT measures the time taken by

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exogenously added thrombin to proteolyze plasma fibrinogen
and to form a clot. TCT/TT assays are not standardized by
the prior art. Each laboratory determines the activity
(strength) of thrombin to be used in the assay. It is

5   customary to adjust the thrombin activity to give a
clotting time of 8 to 9 seconds with 0.2 ml citrated pooled
normal plasma (PNP). This is equivalent to 1.2 NIH units
of thrombin activity.

The usefulness of TCT/TT as a screening assay is

10 underestimated since it is not specific for any disease
condition. However, routine use of TCT/TT alongside the PT
and APTT is invaluable to differentiate efficiencies of the
intrinsic pathway from heparin, and separate lupus-like
anticoagulants from afibrinogemia and paraproteinemia.

15   (7) Euglobulin Lysis Time Test. This is a
crude but effective screening test for accelerated
fibrinolysis. If the clot dissolves much faster than the
control plasma, it is correct to attribute the patient's
condition to a deficiency of the major inhibitor of

20 plasmin, alpha-2-AP or to a deficiency of the major
inhibitor of plasmin, alpha-2-AP or to a deficiency of
activated Factor XIII, the fibrin cross linking
transglutaminase. The concentration of alpha-2-AP in
plasma is half the capacity of plasma for forming plasmin.

25 Therefore, conditions of accelerated fibrinolysis do not
necessarily imply a genetic deficiency of alpha-2-AP, but
could mean a transient depletion of the inhibitor.

Figure 1 shows the possible diagnosis based upon
the AP, T and P tests. All of these tests are performed if

30 there is any abnormal bleeding or a thrombosis.
Other Assays:
(8) Fibrinogen Assays

The unique features of this assay are:
1) it is a qualitative as well as a quantitative

35 thrombin time clotting assay;

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2) serial dilutions of patient and control plasma are performed simultaneously and clotted with thrombin;

3) fibrinogen in PNP has been quantified by Lowry's assay; and

4) Slopes of curves for control PNP and patient plasma are compared. Calculations for patient fibrinogen levels are read off a regression line. The prior art assays:

Prior Art Assays:

1) are based on Clauss method (citation).

2) evaluated patient fibrinogen duplicate clotting times of one plasma dilution. Calculations are made from a semi-logarithmic plot of the regression line of a standard curve performed with purified fibrinogen.

Clinically, the most significant fibrinogen disorders result from:(a) impairment of the velocity with which fibrinogen converts to fibrin and coagulates in plasma; (b) increases or decreases of levels of circulating fibrinogen.

Determination of fibrinogen has, throughout the years, been performed with a variety of methods, each in itself burdened with its own inherent limitations. A brief listing of various methods includes the turbidimetric estimation of fibrinogen after salting out procedures, estimation of fibrinogen by zone electrophoresis, spectrophotometric assays of the purified protein, assays of fibrinogen as fibrin after coagulation, and gravimetric and heat precipitation.

For clinical testing in the present invention, two procedures are performed:

A. Thrombin Clottable Fibrinogen (TCF) Assay, and

B. Heat Precipitatable Plasma Fibrinogen (HPPF) determination by Lowry's Assay.

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A. Thrombin clottable fibrinogen assay (TCF)

In the TCF Assay, a thrombin solution (1.2 - 1.5 NIH units; CT 7-8 secs) is added to serial dilutions of patient plasma and pooled normal plasma (PNP). The
5   dilutions are done with defribilated PNP. Enzyme concentration is always constant; substrate is serially diluted. The defibrilated PNP will be partially corrected by the normal PNP on a reproducible basis. The velocity of fibrinogen conversion and fibrin polymerization is recorded
10 on a coagulation analyzer. Two graphs are sketched by plotting the points for the clotting times of the patient and PNP as shown in Figures 19 to 21. The plasma dilutions are preferably on the x-axis and the clotting times in seconds on the y-axis. By finding the slope and
15 y-intercept of both lines of the graph the following information can be derived:

(1) If the slopes of the line of the patient and the control are parallel; and the y-coordinates (y-intercept mean clotting time) are at the same place on
20 the axis, the diagnosis is that levels of biologically functional fibrinogen are within the normal range.

(2) If the slopes of the line of the patient and the control are parallel but the y-coordinate of the patient is translated upward (more prolonged clotting time)
25 the diagnosis is that levels of biologically functional fibrinogen are decreased.

(3) If the slopes of the line of the patient and the control are parallel but the y-coordinate of the patient is translated downward (shorter clotting times) the
30 diagnosis is that levels of biologically active fibrinogen are increased.

(4) If the graph of the patient has a different slope from that of the control the only possibilities are: heparin or an abornmal fibrinogen. Heparinized plasma will
35 give a false positive result for dysfibrinogenemia.

To rule out the interference by heparin, 1 ml of the patient's plasma is reacted with a heparin absorbent

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chemical (Hepasorb™) (need source) for 10-20 minutes. The
absorbant approach is less costly and just as effective as
the better known reptilase test (citation).

5   Following treatment of patient plasma with
absorbant, the TCF assay is repeated. If the slopes of the
graph continue to be different, an abnormal fibrinogen is
verified by HPPF determination. The diagnostic findings
for dysfibrinogenemia are: discrepancy in levels of
fibrinogen obtained by the HPPF assay (higher) and the TCF
10   assay (lower).

Determination of Fibrinogen Levels

          The points for the fibrinogen levels and
clotting times of control plasma are entered on the
keyboard of a scientific calculator. The slope,
15   y-intercept and the correlation coefficient of the linear
regression line are obtained. To calculate the level of
fibrinogen in the patient's plasma enter a y'-coordinate (a
clotting time for one of the patient plasma dilutions) into
the keyboard. The calculator will find and print the
20   corresponding x-coordinate (control plasma (PNP) dilution)
equivalent to the fibrinogen level for the patient.

Materials and Methods

                    PREPARATION OF PLASMA

Pooled Normal Plasma (PNP)

25   PNP is prepared by pooling human citrated plasma
obtained from 40 healthy volunteers. The PNP can be stored
in 0.5 ml aliquots in small conical plastic tubes at -70°C
and has a stability of 5 years.

Defibrinated Pooled Normal Plasma (Defibr PNP)

30   Pooled Normal Plasma (PNP) for defibrination is
prepared by pooling ten units of outdated citrated human
plasma. The pooled plasma is aliquoted into 25 ml conical
plastic centrifuge tubes. Plasma is defibrinated by
placing the plastic centrifuge tubes containing the plasma
35   in a water bath heated to 56°C. The temperature of the
plasma is brought to 56°C and maintained at that
temperature for 5 minutes. This process denatures

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fibrinogen. Centrifugation at 2,000 rpm for 15 to 20 minutes then precipitates the denatured fibrinogen to the bottom of the tubes.

5   The supernatant is the defibrilated PNP that is used as the diluent in the TCt Assay. A thrombin clotting time is performed to verify the completeness of removal of fibrinogen. Packaging is by placing 1.5 - 2 ml of defibrilated PNP into plastic tubes. Storage is at -70°C and the stability is 10 years.

10   Chemical estimation of fibrinogen:

The denatured fibrinogen pellet is reconstituted in 25 ml distilled water. A Lowry's Assay is performed as described hereinafter. The concentration of fibrinogen in plasma is expressed in milligrams per deciliter.

15   Patient Plasma

Blood is collected in sodium citrate. Quantity: 5 ml blood to yield about 2ml plasma. Patient's plasma is not debrinated, and is used in the assay as whole decalcified plasma.

20   REAGENTS

Tissue Thromboplastin: prepared from rabbit brain.

Calcium Chloride: 0.02M.

Activated Phospholipid Reagents: activating
25   agents can be ellagic acid, kaolin, silica or soybean extract, etc.

Thrombin Reagent: the powdered thrombin is reconstituted in a solution of $CaCl_2$ 0.1M. Clotting activity of thrombin is adjusted to 1.2 unit/per 100ul
30   which will give when added to 200ul PNP a clotting time of 8-9 seconds.

                              EQUIPMENT

Fibrometers or photo optical systems.

35

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Assay Procedures

EXPERIMENTAL PROCEDURES

Plasma Dilutions

Two duplicate sets of fibrocups or cuvettes are
labeled 1 to 6. Pipette in all the cuvettes or fibrocups
200ul defibrilated PNP. Label one duplicate set PNP and
the other duplicate set Patient.

In #1 PNP pipette 200ul PNP perform a serial
dilution of PNP in Defibr PNP. Discard 200ul from #6.

In #1 Patient pipette 200ul patient plasma.
Perform a serial dilution of patient plasma in Defibr PNP.
Discard 200ul from #6.

Measurement of Clot Formation

A. A prothrombin time (PT) assay or a thrombin
clotting time (TCT) assay can be performed. The
clotting times are recorded on the report chart (Figure 7).

Analysis of the Properties of Plasma Fibrinogen

This can be performed on a scientific
calculator, on a personal computer with graphic
capabilities, or on a photo optical coagulation analyzer
with graphics capabilities.

Enter on the keyboard two separate sets of
results:

y: clotting time of plasma dilutions of PNP.
y': clotting time of plasma dilutions of
patient.

x: plasma dilution of PNP
x': plasma dilution of patient
Figure 20 shows the results of the assay with
thrombin. Figure 21 shows the results with prothrombin.

B. Heat precipitatable plasma fibrinogen
determination by modified Lowry's Assay.

Test Procedure:

1. Percent plasma fibrinogen dilutions are made
in the following way:

100% PNP is pooled normal plasma.

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50% PNP is 1ml 100% PNP and 1 ml distilled water.

25% PNP is 1 ml 50% PNP and 1 ml distilled water.

12.5% PNP is 1 ml 25% PNP and 1 ml distilled water.

6.2% PNP is 1 ml 12.5% PNP and 1 ml distilled water.

3.1% PNP is 1 ml 6.2% PNP and 1 ml distilled water.

2. Dilution sets are made up of 1 ml of each plasma fibrinogen dilution. Dilution sets are then incubated separately in a 56°C water bath. Two dilution sets are incubated for 5 minutes; another two dilution sets are incubated for 30 minutes. Centrifugation of each dilution set at 2,000 rpm for 15 minutes immediately after incubation yields the fibrinogen precipitate.

3. Each precipitate is then reconstituted with 1 ml distilled water and determined by Lowry's Assay which is described hereinafter.

In the Lowry's assay, proteins including fibrinogen (5-100 micrograms sensitivity range) are measured with the Folin Phenol Reagent after alkaline copper treatment. Following alkaline treatment, free tyrosine and tryptophan residues react with copper, resulting in a 3- to 15-fold increase in color. This reaction is complete in 5-10 minutes at room temperature. The copper-treated protein will reduce the phosphomolybdicphosphotungstic (Folein) reagent. The final color is greatly enhanced. This reaction is complete in 30 minutes at room temperature.

Reagents

Reagent A: 2% Na_2CO_3 (10 g/500 ml) in 0.10 M NaOH (2 g/500 ml).

Reagent B: 2% Na tartrate and 1% $CuSO_4$ (1:1 volume).

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Reagent C: Alkaline Copper solution: 100 ml A and 2 ml B. (Discard after 1 day).

Reagent E: Folin reagent 2N diluted 1:2 in distilled water to make 1N solution.

5 Procedure:

The sample is 0.2 ml to which 1 ml of Reagent C is added and vortexed immediately.

The sample with Reagent C sits at room temperature for 10 minutes and then 0.1 ml of Reagent E is
10 added and vortexed immediately.

The sample then sits at room temperature for 30 minutes and is then read on Spectrophotometer at 750 nm.

Calibration Curve:

The curve prepared by dissolving 1 mg albumin in
15 1 ml distilled water (1 microgram/1 microliter).

Blanks:

The blanks are:

Distilled water 0.2 ml

Reagent C: 1 ml
20 Reagent E: 0.1 ml

PROCEDURE FOR CALIBRATION CURVE

Albumin 1 mg/1 ml (1 ug/1 ul)

5 ul

10 ul
25 20 ul

50 ul

100 ul

The albumin solution is q.d. to 0.1 or 0.2 ml in distilled water or buffer.
30 (Disposable glass tubes and duplicate samples).

Blanks: 0.1 to 0.2 ml distilled water or buffer.

Protein sample: 5-10 ug sensitivity range.

Add 1 ml of Reagent D and vortex immediately.

The sample then sits at room temperature for ten minutes.
35

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PROCEDURE

Prepare Folin reagent.

      Pipette 3 ml Folin Reagent and 3 ml distilled water to produce 6 ml. Combine in small beaker. Add 0.1
5 ml Folin Reagent to:

      (1) Standard curve; (2) Blank; and (3) Protein samples and vortex immediately.

      The sample then sits for 30 minutes at room temperature and the optical density of samples is read on a
10 spectrophotometer (which can be single or double beam).

SPECTROPHOTOMETER

Set at 750 nm (visible)

1. Warm instrument for 30 minutes prior to use.
2. Calibrate with BLANK
15 3. Use quartz cuvettes (washed in chromerge acid(?))
4. Read optical density (O.D.) of calibration curve)
5. Read O.D. of protein samples
6. Construct on calculator linear regression for calibration curve.

20

      Estimation of slope, intercept and protein concentration of unknown samples can be obtained by entering the:

      Concentration (x values) of albumin used in the
25 calibration curve, followed by entry of O.D. (y values) obtained on spectrophotometer.

Procedure:

      Press: 2nd Pgm 1 SBR CLR to clear the calculator. Then

30

      Press: Value of x   (5 mg)
      Press: x = tg   Then
      Press: value of y   (O.D. reading)
      Press: 2nd   +   Then

      Repeat process for values of x: 10 micrograms,
35 30 micrograms, 50 micrograms, 100 micrograms.

To calculate:

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The y intercept of the line fitted to the data points, PRESS 2nd OP 12. The slope of the line, PRESS 2nd OP 12 x $\equiv$t. The correlation coefficient, PRESS 2nd OP 13. The linear estimate of x on the regression line, enter y value on the keyboard, followed by 2nd OP 15.

The amount of fibrinogen is related to an equivalent amount of the albumin and is expressed in mg/ml. The modified Lowry's assay produces results which are compared to the TCF assay.

The results are charted as set forth in Figure 16.

(9) Inhibitor Assays

Until about five years ago the best known of the coagulation inhibitors were those that developed in hemophiliacs in response to Factor VIII replacement therapy. Nowadays the lupus-like anticoagulant or lupus-like inhibitor has taken in the scientific literature a place of considerable importance. There are several types of lupus-like inhibitors.

Some are purely in vitro phenomena. They are easily identified in phospholipid-dependent coagulation tests. They cause significant prolongation of the clotting times of normal plasma when equal amounts of lupus inhibitor plasma are added to the reaction mixture. Also, plasma with lupus inhibitor does not correct the prolonged clotting times of assorted single factor deficient plasma reagents. Significantly in vitro type lupus inhibitors are neutralized by Platelet Factor III activity.

Other lupus inhibitors have been found to interfere with the activation of coagulation factors and impair the in vivo biological function of these factors. With this type of lupus inhibitor a bleeding diathesis may develop under challenge to the hemostatic system. More often however, they tend to cause an increase in the susceptibility to thrombosis. Lupus inhibitors directed against the biological activity of Factors V, VIII and prothrombin have been identified. These were exclusively present in elderly females with connective tissue disorders.

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Inhibitors have been identified in other categories of
individuals and in association with a variety of diseases.
This type of lupus inhibitor is best identified in mixing
studies with PNP and single factor deficient plasma
.5   reagents as indicated in the chart (Figure 3) labeled
"Inhibitor Screen". The method for the Inhibitor Screen is
very simple. Mix 50ul PNP or single deficient plasma
reagent and perform a PT or an APTT assay. The activator
substance in the APTT assay should be kaolin, soybean
10   extract, silica, never ellagic acid. If evidence points to
a specific coagulation factor inhibition, an inhibitor
assay is performed.

The only difference between in vivo lupus-like
anticoagulants directed specifically against the activity
15   of a coagulation factor and the inhibitors that develop in
hemophiliacs is our lack of our understanding of the site
of action of lupus-like anticoagulants. The inhibitor
Assay can accurately measure the antibody titer of
inhibitors to Factors VIII or Factor IX that develop in
20   hemophiliacs as well as the in vivo lupus-like
anticoagulants. By definition an antibody titer is the
amount of antibody that can bind to one unit of antigen.
Unit can be in measures of weight or activity. In our
assay we measure the inhibition 1 unit of activity of a
25   single coagulation factor.

INHIBITOR ASSAY

Specificity of the inhibitor assay is for:

1) Inhibitors that develop in hemophiliacs and
other individuals. These types of inhibitors are
30   antibodies directed specifically against a procoagulant
factor.

2) Lupus like inhibitors. These are inhibitors
directed specifically against phospholipids. These
antibodies are recognized by their effect on the clotting
35   times of the APTT assay. Two types of lupus like
inhibitors can develop:

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i) a non-specific type that reacts in vitro
with phospholipid reagents to inactivate them. These
inhibitors do not cause bleeding problems.

ii) a specific type that reacts with platelet
5. phospholipids and prevents complex formation and assembly
These inhibitors can cause serious bleeding problems.

The principle of the inhibitor assay for
inhibitors that develop in hemophiliacs and other
individuals is a direct inhibitor assay similar to the
10 passive hemaglutination assay. The end point of this assay
determines the amount of factor activity inhibited by a
known quantity of patient plasma. Thereby calculations are
made as to how much factor concentrate is needed to
overcome the inhibitor.

15 The principle of the assay for lupus like
inhibitors is to mix in equal proportions of the patient's
plasma and a control plasma. If the prolonged clotting
times by the APTT assay are corrected, a lupus like
inhibitor can be ruled out with certainty. To provide
20 proof for a lupus like inhibitor, the patient's plasma is
mixed in equal proportions with Factor VIII deficient
plasma and an APTT assay is performed. If the prolonged
clotting time by the APTT is not corrected, evidence is
very strong of a lupus like inhibitor.

25 Reagents:

Citrated pooled normal plasma (about 1 ml).
Factor VIII deficient plasma (about 2 ml)
Activated Partial Thromboplastin Reagent
("Actin™"), purchased from American Dade (ellagic acid), or
30 Helena™ APTT Reagent (kaolin) $CaCl_2$ (0.02M)
MLA pipettes and pipette tips: 200 microliters,
50 microliters, 30microliters, 20microliters and 10
microliters.

Method

35 Step 1:

Prepare 5 tubes, label 1-5
Tube #   Plasma Mixture (Antigen dilution, FVIII deficient)

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1   200 microliters PNP

2   200 microliters PNP + 200 microliters FVIII
         deficient. Mix.

3   Add 200 microliters from tube 2 + 200 microliters
         FVIII deficient. Mix.

4   Add 200 microliters from tube 3 + 200 microliters
         FVIII deficient. Mix.

5   Add 200 microliters from tube 4 + 200 microliters
         FVIII deficient. Mix and discard 200 micro-
         liters.

6   200 microliters patient's plasma.

Step 2: Add inhibitor to antigen dilution.

Tube 1   0.16 unit FVIII/200 microliters, add 50 micro-
           liters patient plasma.

Tube 2   0.08 unit FVIII/200 microliters, add 30 micro-
           liters patient plasma.

Tube 3   0.04 unit FVIII/200 microliters, add 20 micro-
           liters patient plasma.

Tube 4   0.02 unit FVIII/200 microliters, add 10 micro-
           liters patient plasma.

Tube 5   0.01 unit FVIII/200 microliters, add 10 micro-
           liters patient plasma.

Control: Standard curve of PNP in Factor VIII deficient
          plasma.

Label tubes 1-10.

    80%   1. 200 microliters PNP

    40%   2. 200 microliters PNP + 200 microliters
                 FVIII deficient. Mix.

    20%   3. Add 200 microliters from tube 2 to 200
                 microliters FVIII deficient. Mix.

    10%   4. Add 200 microliters from tube 3 to 200
                 microliters FVIII deficient. Mix.

    5%   5. Add 200 microliters from tube 4 to 200
                 microliters FVIII deficient. Mix.

    2.5%   6. Add 200 microliters from tube 5 to 200
                 microliters FVIII deficient. Mix.

    1.2%   7. Add 200 microliters from tube 6 to 200

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microliters FVIII deficient. Mix.

0.6%   8.   Add 200 microliters from tube 7 to 200
            microliters FVIII deficient. Mix.

0.3%   9.   Add 200 microliters from tube 8 to 200
            microliters FVIII deficient. Mix.

0.1%   10. Add 200 microliters from tube 9 to 200
            microliters FVIII deficient, mix and
            discard 200 microliters.

Step III:

Incubation: Plasma mixtures are incubated for 1
hour at 37°C.

At the end of the incubation period, 100
microliters of plasma mixture from each tube is added to
100 microliters prewarmed activated partial thromboplastin
reagent in fibrocups. After 3 minutes, the mixture is
clotted with 100 microliters CaCl₂ 0.02M.

Calculations

Follow the instructions outlined on the report
chart Figure 12. Results are interpreted as follows:

1. A powerful inhibitor will overcome and
neutralize all of the activity present in all plasma
dilutions, and consequently one is unable to determine the
antibody titer. In this instance the test should be
repeated using 20 ul, 10 ul, 5 ul and 1 ul of patient's
plasma while preserving the same initial factor activity in
the plasma mixture.

2. In another case, 1 ml of patient's plasma
inhibits 3 units of Factor VIII activity Since an adult
human of average weight and height will have 3 liters of
circulating plasma, it is probable that 9,000 units Factor
VIII concentrate will neutralize the inhibitor _in vivo_.

3. A third example is that of a lupus-like
anticoagulant. When tested in this system it will
neutralize any of the plasma activity in the mixture.

(10) Antithrombin III Assays

The unique features of the Antithrombin III
assay are: 1) it is a clotting assay; 2) the plasma is not

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diluted and is defibrinated; 3) the thrombin used in the
assay is standardized; 4) heparin added to the assay
mixture is standardized; 5) fibrinogen used in the assay is
ATIII deficient plasma fibrinogen (200 micrograms per 100

5   microliters); 6) calculations are based on molecular
interaction of ATIII and thrombin in the inactivation
process.

          In all other ATIII clinical clotting assays: 1)
plasma is variably diluted; 2) thrombin activity is not

10   recorded or has not been determined; 3) heparin is
standardized; 4) purified fibrinogen is used for the end
point; 5) calculations are based on a standard curve of
thrombin fibrinogen interaction.

          ATIII levels are decreased in hereditary ATIII

15   deficiency, intravascular coagulation, hepatitis and
hepatic cirrhosis and chronic nephritis. Significant
laboratory findings in acute disseminated intravascular
coagulation are low levels of ATIII activity measured by
clotting assays, low platelet count, and decreased levels

20   of fibrinogen. Hereditary ATIII deficiency is
characterized by reduced ATIII activity and normal or
reduced concentrations of ATIII measured by immunological
assays. Other hemostatic parameters are normal.

          In clinical laboratories, both clotting and

25   amidolytic assays are used to evaluate the heparin cofactor
activity of ATIII. It has been found that ATIII
selectively inhibits the clotting activity of thrombin. In
other words, when ATIII first forms a complex with thrombin
the inhibitor binds the enzyme at the fibrinogen binding

30   site so that even though the clotting activity of thrombin
is inhibited, complexed thrombin has the ability to
hydrolyze chromogenic substrates. It has been found that
the non-clotting forms of thrombin or "autolyzed thrombins"
(beta and gamma thrombin) also form complexes with ATIII.

35   In commercial thrombin preparations, the autolyzed
thrombins are present in higher proportion than the
clotting thrombin "autolyzed thrombins" have the ability to

hydrolyze chromogenic substrates as well as to bind ATIII
but do not hydrolyze fibrinogen. Therefore, chromogenic
substrate assays measure clotting thrombin forms, thrombin
complexed with ATIII as well as autolyzed thrombins. The

5   use of such assays in clinical testing is questionable.
The clinical significance of ATIII lies in its ability to
inhibit the clotting form of thrombin from hydrolyzing
fibrinogen to fibrin.

       ATIII levels are measured in the present

10   invention by a clotting assay and an immunologic assay.
Chromogenic substrate assays are never used.

       To devise an assay to measure the heparin
cofactor activity of ATIII in plasma, several very
important facts about the effect of heparin on coagulation

15   proteins were taken into account.

       Heparin sodium is a mixture of active principles
having the property of prolonging blood clotting. The
route of administration is intravenous or subcutaneous.
Its mode of action is to bind to ATIII. The greater impact

20   of heparin is its _in vivo_ effect on ATIII. This effect is
known as the anticoagulant effect of heparin. The
discovery of heparin-ATIII interaction provided an
explanation for the long-known action of heparin. There is
evidence that heparin interacts with ATIII by electrostatic

25   binding to lysine residues of the ATIII molecule. In the
presence of heparin, the preferential target of ATIII
appears to be thrombin. Some subspecies of heparin may
enhance the interaction of ATIII with Factor Xa more than
with thrombin or vice versa. When formation of the

30   ATIII-thrombin complex has occurred, heparin readily
dissociates from the complex. Thus, it acts as a catalyst
of the neutralizing process.

       However, the following facts though less well
known are equally important.

35   1. _In vitro_ heparin has not one but two actions
on blood coagulation. The less known one is a _direct_
inhibitory effect. Heparin inhibits thrombin and Factor Xa

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by a direct effect that is due to electrostatic attraction.
The inhibition involves the formation of reversible
complexes that interfere with the procoagulant effect of
both enzymes. Thus heparin has an anticoagulant and·
5   antithrombotic effect. The antithrombotic effect of
heparin is .one of the side effects of heparin therapy.

2. At low heparin concentration, fibrinogen
acts as an antithrombin, apparently due to an induced
change in the charge of fibrinogen by heparin. This effect
10   is absent in defibrinated plasma and in serum and is
significant when the ATIII/heparin cofactor activity of
plasma is evaluated.

For clinical testing two procedures are
performed:
15   ATIII/heparin cofactor activity assay.
Counter electrophoresis or cross over.
Immunoelectrophoresis (cross over IEP) immuno
assay.

In the ATIII/Heparin Assay:
20   (1) Test plasma (patient and PNP) is heat
defibrinated. This is done to eliminate the effect of
fibrinogen on heparin.

(2) Low concentrations of heparin are used: 0.3
- 0.4 unit/ml. The purpose is to eliminate the direct
25   effect of heparin on thrombin.

(3) Patient and control PNP are never diluted.
Again, here the purpose is to eliminate the direct effect
of heparin on thrombin. When plasma ATIII is greatly
diluted thrombin inhibition will occur as a result of
30   inactivation by ATIII but also as a result of direct
inactivation by heparin. Electrostatic binding of the
negatively charged heparin to the active site of the
thrombin will prevent the enzyme from hydrolyzing
fibrinogen. Thus falsely high values for ATIII levels can
35   be obtained.

(4) Thrombin concentration is calculated to be
less than plasma ATIII level in the test sample.

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PREPARATION OF REAGENTS

1. Patient plasma and control PNP are heat defibrinated by the method described in the fibrinogen assay.

A thrombin solution (1.2 to 1.5 NIH units/100ul) to give a clotting time of 7-8 seconds when added to 200ul PNP is prepared in 0.1M CaCl$_2$ in one single 15ml batch. Thrombin is not frozen and is stored in a plastic tube at 4°C in the refrigerator. The activity lasts for over 3 to 4 months or even longer if properly handled. Small aliquots for the assay are used at room temperature.

3. A heparin solution is prepared in distilled water as follows: take from the stock solution <u>one unit</u> of heparin. Add this <u>one unit</u> to 10ml distilled water, mix gently. Heparin concentration is 0.1 unit/100ul. To titrate the heparin activity suitable for the assay the following should be done.

a. take 5-6 fibrocups or cuvettes and place 200ul PNP.

b. take MLA pipettes calibrated 20, 30, 40, 50, 60ul.

c. add 20ul heparin solution to plasma in fibrocup and clot with thrombin. Record clotting time. Repeat with 30ul, 40ul, etc.

d. ideal clotting time for the assay is 30-45 seconds.

ASSAY PROCEDURE

The results are reported in the chart (Figure 14).

1. 100ul defibrinated patient plasma are placed in 4 fibrocups.

2. 100ul defibrinated PNP plasma are placed in 4 fibrocups.

3. Add heparin to 2 fibrocups. Add thrombin then clot with PNP (100ul). Record clotting time.

4. Other 2 fibrocups, add thrombin then clot with PNP.

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CALCULATIONS

The stoichiometry of the inactivation of
thrombin by ATIII is 1:1. The clotting assay for
measurement of ATIII is an indirect assay that reflects the
5   loss of thrombin activity. Therefore, the ATIII levels
are calculated indirectly by calculating the loss of
thrombin activity. From Figure 23, the ATIII levels can be
obtained from the clotting times.
(11)

10   IMMUNOLOGICAL ASSAY

HEPARIN ASSAYS

The unique features of the heparin assay are
that:

(1) the sensitivity and specificity of the PT,
15   TCT, and APTT for heparin at therapeutic ranges; (2) the
specificity and sensitivity of heparin assays are not
conferred by the heparin source (beef or porcine), or the
purity of the thrombin reagent; (3) powdered heparin is
more stable than liquid reagents; (4) computerized standard
20   curves for heparin assays can be obtained.

Heparin assays in other laboratories:

(1) The APTT assay is the most widely used.
APTT is sensitive to heparin but cannot measure heparin
levels in plasma;(2) There is widespread confusion as to
25   the sensitivity of heparin assays using different animal
sources and curves are prepared fresh each day.

In the present invention, test sensitivity to
heparin was measured for three tests: the PT, APTT, and TCT.
Their sensitivity to both liquid and powdered heparin was
30   measured. The experimental condition and procedures were
the same for both types of heparin.

Historically, therapeutic heparin ranges were
described as the amount of heparin that will prolong the
whole blood clotting time to twice baseline value by the
35   Lee and White assay (citation). Later, clinical trials
were conducted with heparin on patients with thrombotic
disease. It was then determined that plasma levels of 0.2

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to 0.4 unit/ml heparin were sufficient to keep the patients
anticoagulated. When heparin was administered at higher
doses it was found that plasma heparin levels of 0.5
unit/ml or higher resulted in increased bleeding tendency.
5   Heparin plasma levels of 0.2 unit/ml were not adequate to
check the thrombotic process.

        At the present, the Lee and White clotting time
assay is rarely performed. The most widely used laboratory
procedure for monitoring heparin therapy is the APTT. The
10 TCT is considered by some to be a more reliable test.
However, there are no reports on the use of the PT assay
for monitoring heparin.

Powdered heparin:

        2 mg of 162 units/mg powdered heparin (sodium),
15 obtained through Sigma Chemical Co., St. Louis, Mo., was
added to 32.4 ml of distilled water. This stock solution
was reconstituted form each test to a concentration of 0.1
unit/ml.

Liquid heparin:

20   10 ul of liquid heparin (sodium), obtained
through Elkins-Sinn, Inc., Cherry Hill, N.J., was added to
10 ml of distilled water. This solution was then
reconstituted to a concentration of 0.1 u/ml.

Test Procedures:

25   All tests were performed on Helena Laboratory's
Dataclot-2™ fibrometer using pooled normal plasma obtained
from 40 healthy donors age 18 to 64 stored at a temperature
of -70°C. All plasma and reagents were transferred using
MLA pipettes.

30   Thromboplastin-C (Rabbit brain) obtained from
American Dade AHS del Caribe, Inc., Aguada, Puerto Rico,
00602, was used as the reagent for the PT tests.

        For the TCT test, thrombin was prepared to give
an activity of 1.2 NIH unit.

35   The PT and TCT were performed 10 times on pooled
normal plasma with no heparin added to obtain baseline
clotting times. Then, 10 ul of .1 u/ml heparin was added to

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the plasma and four clotting times were obtained. Then, 20 ul of .1 u/ml heparin was added to the plasma and four clotting times were obtained. Each new concentration of heparin was increased by 0.1 u/kml increments in this manner until the clotting times became so long that accurate data collection was no longer possible.

The entire procedure was repeated three times, each time with newly reconstituted heparin and other reagents.

The APTT reagents were comprised of Thrombosil-I (Rabbit brain cephalin and silica activator) Lot 3 1TH111 and Calcium Chloride Solution (0.02 $\underline{M}$ aq.) Lot # CAL 300. Both were obtained from Ortho Diagnostic Systems, Inc., Raritan, N.J. 08869.

The APTT test was performed in the same manner with the exception that the initial heparin concentration was 0.05 u/ml and each new heparin concentration value was increased by 0.05 u/ml, rather than by 0.1 u/ml as in the PT and TCT test procedures.

A representative graph is shown in Figure 23. Figure 14 shows the chart for recording antithrombin III.

(12) <u>Protein C</u>

Protein C is the zymogen of a serine protease, Protein C_A. Protein C_A exerts an anticoagulant effect in plasma by the selective inactivation of non-enzymic activated cofactors FVa and FVIIIa. It has been shown by several investigators that native Factors V and VIII are poor substrates for Protein C_A. It has also been shown that on endothelial cell surfaces in blood, Protein C is activated to a protease by thrombin complexed with thrombomodulin. Thrombomodulin is an integral endothelial cell surface protein.

In <u>vitro</u> Protein C is slowly activated by thrombin alone or by thrombin/thrombomodulin at a much faster rate. Also Protein C is activated by purified Factor X_a and by Akistrodon Akistrodon Contortrix venom of the Southern Copperhead snake. The component in the

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Akistrodon venom that is selective for the activation of
Protein C has been purified and is given the trade name
"PROTAC"® (available from Sigma Chemical Company, St.
Louis, MO.). In addition to activating Protein C, the
5   purified component of Akistrodon Akistrodon Contortrix has
been found to decrease, by direct proteolysis, the
procoagulant activities of purified Factors II, VII, IX, X,
and to cleave the A alpha chain of fibrinogen
(Thrombomodulin Activityin Commercial Thromboplastin
10 Preparations. Thrombos Res. 43, 265-274 (1986)). Thus, in
vitro, the Protein C activator from the Southern Copperhead
snake venom exerts a broad substrate specificity.

        Several assays to measure the biological
activity of Protein C in plasma have been published. Some
15 utilize lengthy and rather complicated experimental
procedures that preclude their use in clinical diagnostic
laboratories. Others use the purified Protein C activator
from Akistrodon venom to measure Protein C activity as a
function of the prologation of the APTT clotting times.
20 A.   Principle of the Protein C Assay

        Plasma is activated by thrombomodulin/tissue
Factor and calcium chloride.

        Amount of tissue Factor and calcium chloride
that activate plasma was carefully calculated. As little
25 as 20 ul of commercial ThromboplAstin/CaCl$_2$ 0.02M solution
activates PNP. Evidence for generation of thrombin in the
activated plasma is obtained by an increase in Factor V and
Factor VIII activity without detectable fibrin formation.

        The maximum amount of Thromboplastin/CaCl$_2$ 0.02M
30 solution that fully activates PNP without detectable fibrin
formation is 50 ul.

        To prepare a suitable commercial
Thromboplastin/CaCl$_2$ 0.02M solution that will activate
plasma at the recommended 20ul to 50ul range, distilled
35 water is added to the dried powder to give in a fibrometer
a clotting time by the PT assay for PNP of 11.6 seconds

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(0.5). The reagent recommended is Ortho Thromboplastin for
its high thrombomodulin activity.

Measurement of Activated Factors V and VII by
the APTT assay.

5   30ul plasma before activation and 30ul plasma
after activation are each added to 70ul Factor V and Factor
VIII deficient plasma. Measurement of change in activity
is a change in the clotting time by the APTT assay.
Activity is derived from the standard curves presented in
10 the Table shown in Figure 29. The Table of Figure 29 shows
standard curves for descending ranges of Factors V, VIII,
IX, X and XI activities and the mean clotting times
obtained by the APTT assay. A least squares linear
regression of the actual data points from the straight
15 lines of the best fit are shown in Figure 29. Factor
activities and the corresponding clotting that represent
the critical threshold procoagulant Factor V, VIII, IX, X
or XI activities are shown.

Protein C is activated in Activated Plasma by Akistrodon
20 Contortrix

Protein C in Activated Plasma is activated by 80
to 100 ng Akistrodon Akistrodon Contortrix (Southern
Copperhead Venom). The proteolytic activity of snake venom
used, 80 to 100 ng, is selective for Protein C.

25   The activation of Protein C with Akistrodon is
used to speed the process that is started by
thrombin/thrombomodulin $CaCl_2$ as described above. If the
snake venom step is omitted, a time interval of at least 4
hours is necessary for the inactivation of Factors V_a and
30 Factors $VIII_a$ by "thrombin activated Protein Ca".

B. Biological Activity of Protein C_A is measured as a
function of percent change in Factor V and Factor VIII
activity.

At the end of one hour incubation, 30 ul of
35 plasma mixture are added to 70ul of Factor V and Factor
VIII deficient plasma. Factor activity is obtained from
clotting times by means of standard curves in Figure 29.

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MATERIALS AND REAGENTS
Preparation of Pooled Normal Plasma (PNP)

Human pooled normal plasma (PNP) was prepared
from forty healthy blood donors ages 18 to 64 years. Blood
5   (4.5 ml) was drawn from each donor into vacutainer tubes
each containing 0.5 ml of 3.85% acidified sodium citrate
solution. Blood was spun at 2,000 r.p.m. in a refrigerated
Beckman table top centrifuge at 2°C for 10 to 15 minutes.
Platelet poor plasma was pooled into a polystyrene beaker
10 placed on ice. The pooled plasma was assayed for
procoagulant factor levels by the PT and APTT assays.
Fibrinogen levels were determined by clotting and chemical
assays. PNP aliquots (1 ml) were pipetted into 4 ml
polystyrene capped tubes and stored at -80°C for use in the
15 Protein C experiments.

Akistrodon Contortrix Venom (Southern Copperhead venom)

One gram freeze dried venom powder was purchased
from Sigma Chemical Company, St. Louis, MO. Twenty samples
of dried powder were weighed 0.1 mg each and stored in 15
20 ml graduated capped plastic centrifuge tubes at 4°C until
further use. The dried venom was dissolved in distilled
water (0.1mg/10ml) and assayed for stability by adding
500ng/50ul to 1 ml PNP. The proteolytic anticoagulant
activity was tested by the APTT assay. Proteolytic
25 anticoagulant activity was markedly decreased within 24
hours after reconstitution in distilled water. Proteolytic
anticoagulant activity was retained in the dried powder.
Fresh solutions therefore were prepared daily by adding 10
ml distilled water to the graduated plastic centrifuge
30 tubes containing 0.1 mg of dried powder. Venom solutions
were kept on ice for the duration of the experiments.

The venom was also tested at two concentrations
of 80 ng and 500 ng added to 1 ml PNP for substrate
selectivity by PT, APTT and thrombin Clotting Time (TCT)
35 assays.

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Tissue Thromboplastin/Calcium Chloride Powder (TTP/CaCl$_2$)

This was purchased from Ortho Diagnostic Systems Inc., Raritan, N.J. Ortho Brain thromboplastin ISI Standard lot 871007 was obtained from the same source. The
5   commercially prepared tissue thromboplastin/calcium chloride powder was reconstituted to give a clotting time of 11.6 ± 0.5 seconds on 100 ul PNP. This reagent has high thrombomodulin activity (Thrombomodulin Activity in Commercial Thromboplastin Preparations. Thrombos Res. 43,
10   265-274 (1986)).

Activated Partial Thromboplastin Reagent

Thrombosil I, a commercially prepared brain cephalin with silica activator, was purchased from Ortho Diagnostic Systems.

15   Calcium Chloride Reagent

0.02 molar solution and Thrombofax Reagent. A bovine brain cephalin solution were also purchased from Ortho Diagnostic Systems.

Human Alpha Thrombin with a specific activity of
20   3,000 units/ug was prepared. Clotting activity of the thrombin in 0.1 M CaCl$_2$ solutions is retained for several years. A preservative, Thimerosal purchased from Sigma Chemical Company is added to the thrombin solutions at 1/100,000 (weight in mg/volume). Thrombin solutions (1.5
25   to 1.2 unit per 100 ul 0.1 M CaCl$_2$) were prepared to give a clotting time of 8-10 seconds with 200 ul PNP.

Equipment

A Dataclot 2 fibrometer, Helena Laboratories, Beaumont, Texas was used for the clotting experiments. A
30   MacIntosh Apple Computer and an IBM PC were used for the analysis and graphing of the data.

EXPERIMENTAL PROCEDURES

Prothrombin Time (PT) Assay

PNP or plasma mixture (100ul) were clotted with
35   200ul TTP/CaCl$_2$ solution. The clotting times were recorded on a fibrometer.

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Activated Partial Thromboplastin Time (APTT) Assay

PNP or plasma mixture (100ul) were incubated with APTT reagent for 3 to 5 minutes then clotted with 100 ul CaCl2 0.02 M.

5 Thrombin Clotting Time (TCT) Assay

200ul PNP or plasma mixture was clotted with 100ul thrombin solution (1.5 to 1.2 unit).

Single Factor Genetically Deficient Plasma Reagents (less than 1% activity)

10 Factor XI deficient reagent was purchased from George King, Biomedical, Inc., Overland Park, KS. All other factor deficient plasmas were obtained by plasmapherisis from patients at Michigan State University, East Lansing, Michigan.

15 Standard Curves for Factors V, VII, VIII, IX, X, XI were constructed using single factor genetically deficient plasma and PNP. The clotting times by PT and APTT assays for about forty estimates per point were analyzed. Standard deviation, linear regression, Pearson's

20 correlation coefficient, as well as mean and median were calculated for each curve.

In Table I the clotting times by PT for Factors V, VII and X activities ranging from 80% to less than 1% are presented.

25 In Table II the data presented are the clotting times by the APTT assay for Factors V, VIII, IX, X, and XI activities ranging from 80% to less than 1%.

Factor Assays:

In the Protein C assay experiments, most of the

30 Factor assays were performed by PT or APTT assay after adding 30ul plasma mixtures to 70ul single factor deficient plasma (Factors V, VII, VIII, IX, X, XI or XII) and recording the mean clotting times. The mean clotting time was never less than four estimates with an average of ten

35 estimates per point. Factor activities were then derived from the corresponding clotting times on the linear regression of the standard curves.

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Activation of Plasma

          One milliliter of PNP or patient plasma was
activated by adding 50ul, 30ul, 20ul or 10ul solutions of
TTP/CaCl$_2$. Tube was gently shaken and incubated at 37°C
5   for times ranging from zero to one hour. PT, APTT, TCT,
and Factor assays were performed on activated plasma and on
plasma prior to activation.

Standard Curves for Protein C

          Three standard curves for Protein C were
10   contructed in:

          1) Protein C freeze dried deficient plasma
reagent purchased from Diagnostica Stago, Asniere, France.

          2) Plasma obtained froma 16 year-old patient who
tried to commit suicide by ingestion of three packages of a
15   long lasting coumarin derivative prepared commercially and
used as a rat poison (trade name: Enforcer™). PT was 72
secs (control 11.2 secs), APTT 132.4 secs (control 26.4
secs), and TCT 9.9 secs (control 9.2 secs). Factor VII
activity in this patient's plasma was less than 1%, Factor
20   X activity 2%, and FIX activity 2.5%.

          3) PNP immunodepleted of Protein C by
anti-Protein C insolubilized rabbit immunoglobulins.

          Human anti Protein C antibodies were purchased
from Diagnostica Stago Asniere, France. The commercial
25   antibodies were not charcterized in my laboratory for
antigen specificity and cross-reactivity. Coupling of the
anti Protein C antibodies to sepharose beads and immuno
depletion of PNP by insolubilized antibodies was performed
exactly as described (H. I. Hassouna and J. A. Penner. Sem.
30   Thromb. Haemost. Vol. 7, No. 2, pp. 61-111 (1981)).

Patient Tests

          Following surgery for cancer of the pancreas, a
61 year old patient suffered a pulmonary embolism. He was
placed on coumadin, 5 mg/day. Two months later, while
35   still on coumadin, he was hospitalized for spontaneous
bleeding, bruising and a hematoma on the left thigh. He
had lost eight pounds because he was not eating.

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At the time of admission, his PT was 60 seconds, APTT greater than 100 seconds, platelet count 140,000/cc, fibrinogen 425 mg/dl, and fibrin split products moderately elevated. Liver function tests were unchanged from previous records.

He was diagnosed as disseminated intravascular coagulation (DIC), was taken off coumadin and given fresh frozen plasma. His PT and APTT corrected for six hours. For the next three days, his PT and APTT were still prolonged; PT 20-22 seconds (control 11.6 secs.) and his APTT 38 seconds (control 26.4 secs.). Fibrinogen remained unchanged, at 425 mg/dl. Fibrin split products were not ordered.

<u>Discussion</u>

The diagnosis of DIC was made on the basis of a prolonged PT and APTT and a mild elevation of fibrin split products. The fact that fibrinogen levels and platelet counts were within normal range was attributed to a possible decline in initially higher than normal values. Other possible diagnoses were disregarded, owing to the myth that an APTT is never prolonged with coumadin therapy and that a PT of 60 seconds is possible even with fibrinogen levels of 425 mg/dl. Also the specificity of immunoassays for the determination of fibrin split products was never questioned.

In this case, access to the present invention data would have given the clinician the ability to make the correct diagnosis.

1) The Prothrombin Time (PT) test measures Factor VII, as well as Fibrinogen, Factors V, X, and prothrombin. This can be seen in Figure 1 (Coagulation Screening Tests). Also, maximal prolongation of the clotting times by the PT test for a single factor deficiency is less than 50 seconds, provided fibrinogen levels are normal (see Figure 2 top section and Figure 3). Similarly, maximal prolongation of the clotting times by the Activated Partial Thromboplastin Time (APTT) test for

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Factor VIII deficiency is 77-80 seconds (see Figure 2-lower
section and Figure 5). Drugs, such as coumadin, that
interfere with the synthesis of biologically active vitamin
K dependent factors have an effect on the PT as well as the
5   APTT (see Figure 5-4th section from bottom of page). PT or
APTT tests are considered prolonged if the clotting times
are outside the normal distribution. Normal distributions
for both the PT and APTT are indicated in the top section
of Table V and detailed in the histograms marked Figures 6
10   and 7. In DIC, Factors V and VIII are the major
procoagulant factors consumed. The half lives of Factors V
and VIII are 12-36 hours and 2.9 days respectively (see
Figure 8).

          2) When the patient was given fresh frozen
15   plasma, the PT and APTT corrected for six hours then became
prolonged again. The prolonged coagulation times, though
modest, were significant.

          3) We can therefore contemplate one of two
conditions:
20   a) a low grade ongoing disseminated
intravascular coagulation

                    or

                (b) a multiple deficiency involving the
vitamin K dependent factors due to malnutrition and
25   possible malabsorption associated with coumadin therapy.

          4) Confirmatory tests are indicated in table IX
(Diagnosis of Acute Thrombosis). Testing for Antithrombin
III, Protein C, and plasminogen will provide proof for or
refute DIC.

30   Another approach would be to test for Factor V.
If Factor V levels are within the normal range, a diagnosis
for vitamin K deficiency can be made.

Diagnosis

          Vitamin K deficiency due to malnutrition and
35   possible malabsorption associated with coumadin therapy.

          Better diagnostic tests for diseases caused by
abnormal blood clot formation (heart disease, strokes, deep

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clots in lungs, legs) are in demand. These conditions are
an enormous health problem and public awareness of them has
been increasing steadily with growth in health
consciousness. They are life threatening diseases
5   associated with aging, lack of exercise, poor dietary
habits, smoking and oral contraception so they have a lot
of visibility. Thrombotic (clotting) disorders are being
handled now with new treatments, like tPA and oral
anticoagulants, that complicate the interpretation of
10   laboratory tests needed for diagnosis and for monitoring
therapy.

        There is already an established market for
coagulation disease diagnostic kit sales to the
medical-technology community. The fact is though that this
15   traditional technology, based on concepts prevalent in the
1950's, does not provide the quantitative precision that is
essential for this expanding market and for the
complexities that result from modern medical and surgical
management of coagulation diseases. The present invention
20   is a validated, proven system of original assays, founded
on a very extensive data-base of thousands of test results
on samples from diseased and normal subjects. It is
compiled in a comprehensive, differential-diagnostic format
and relies on some preferred reagents with highly desirable
25   characteristics. The system makes high quality information
available to the diagnostician conveniently and quickly,
improving the decision-making process for the clinician.
It can even identify those "at risk" of clot formation and
therefore has the potential for incorporation into
30   screening panels for routine use.

        The preceding description is only illustrative
of the present invention and it is intended that the
present invention be limited only to the hereinafter
appended claims.

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I CLAIM:

-1-

A method for diagnosing blood clotting disorders
in humans which comprises:

(a) separately testing sets of a sample of
plasma separated from the blood of a patient and a set of a
sample of plasma from pooled normal plasma (PNP) from
healthy humans for clotting time (CT) by addition of
predetermined amounts of prothrombin (P) to a first set of
the sample; activated partial thromboplastin (APT) to a
second set of the sample and thrombin (T) to a third set of
the sample, charting the results for the patient and the
PNP together on a side-by-side basis for P, APT and T and
comparing the results with a data base showing normal
ranges of CT based upon the PNP for APT, TT and P wherein
the APT, T and P have been separately prepared in solution
to produce a particular standardized clotting time with PNP
which is used in all of the testing of the patient plasma;

(b) testing for hypercoagulation or bleeding
based upon the P, APT and T tests and charting the results;
and

(c) providing a diagnosis based upon the
differences of CT based upon the tests.

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The method of Claim 1 wherein in addition fourth
sets of the sample of the plasma from the patient and the
plasma from the PNP are tested separately for bleeding by
mixing a volume of a genetic factor deficient plasma (GFDP)
5   with PNP in an amount between about 40 and 60 percent by
volume of GFPD to PNP and patient plasma wherein the
deficiency is selected from the group consisting of Factors
V, VII, VIII, IX, X, XI, VII, Fl.F and HMWK coagulated with
an appropriate one of P or APT or both separately and the
10   CT determined for the mixture, wherein the results for the
CT are charted on a side-by-side basis for the patient and
the PNP, wherein the results are compared with a Factor
data base showing a normal range for each Factor based upon
the PNP and wherein the CT of GFPD is corrected by the PNP
15   and by normal patient plasma.

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The method of Claim 1 wherein in addition the
sample of the plasma from the patient is tested for
antithrombin III (ATIII) and the results compared with a
antithrombin III data base showing normal ranges of CT.

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The method of Claim 1 wherein in addition the
samples of plasma from the patient and the PNP are tested
for protein C in activated form using P or APT and genetic
factor deficient plasma (GFDP) deficient in Factor V or
5   Factor VIII, wherein the results for the patient and the
PNP are charted on a side-by-side basis, wherein the
results compared with a protein C data base showing normal
ranges based upon the PNP and wherein normal patient plasma
inhibits activated Factors V and VIII to produce a
10   prolonged clotting time.

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The method of Claim 1 wherein in addition the
samples of the plasma of the patient and the PNP are tested
for fibrinogen by determining CT using T and multiple
dilutions of the plasma with defibrilated PNP, wherein the
5   results for the patient and the PNP are charted on a
side-by-side basis and wherein the results are compared
with a fibrinogen data base showing normal ranges based
upon the PNP.

10

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The method of Claim 1 wherein in addition the
samples of the plasma from the patient and the plasma from
the PNP are tested separately for bleeding by mixing with
known volumes of a genetic factor deficient plasma (GFDP)
5   with PNP in an amount between 40% and 60% by volume patient
plasma to PNP wherein the deficiency is selected from the
group consisting of Factors V, VII, VIII, IX, X, XI, VII,
F1.F or HMWK coagulated with an appropriate one of P or APT
or both separately and the CT determined for the mixture,
10   wherein the results for the CT are charted on a
side-by-side basis for the patient and the PNP and wherein
the results are compared with a Factor data base showing a
normal range for each Factor based upon the PNP and wherein
the CT of GFDP is corrected by PNP and normal patient
15   plasma;

wherein in addition the sample of the plasma
from the patient is tested for antithrombin III (ATIII) and
the results compared with an antithrombin III data base
showing normal and abnormal ranges;
20   wherein in addition the samples of plasma from
the patient and the PNP are tested for protein C in
activated form using P or APT and GFDP deficient in Factor
V or in Factor VIII, wherein the results for the patient
and the PNP are charted on a side-by-side basis, wherein
25   the results compared with a protein C data base showing
normal ranges based upon the PNP and wherein normal patient
plasma inhibits activated Factors V and VIII to produce a
prolonged clotting time; and

wherein in addition samples of the plasma of the
30   patient and the PNP are tested for fibrinogen by
determining CT using T and multiple dilutions of the
plasma with defibrilated PNP, wherein the results for the
patient and the PNP are charted on a side-by-side basis and
wherein the results compared with a fibrinogen data base
35   showing normal ranges based upon the PNP.

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The method of Claim 6 wherein the results are combined on a single chart for bleeding disorders or for hypercoagulation.

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The method of Claim 1 wherein the data base is maintained in a computer.

-9-

The method of Claim 1 wherein the charts are maintained as screens on a computer which can be completed for each patient and optionally printed out on a printer connected to the computer.

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The method of Claim 1 wherein the data base is maintained in a computer and wherein the charts are maintained as screens on the computer which are completed for each patient as the tests are performed.

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The method of Claim 1 wherein a computer program provides a basis for differential diagnosis based upon the CT for P, APT and T and suggests further tests for hyperclotting or excess bleeding.

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The method of Claim 6 wherein the data bases are provided on a program in a computer.

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The method of Claim 12 wherein the charts are maintained as screens on the computer which are completed as the tests are performed.

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The method of Claim 13 wherein the computer integrates the test results from the data bases to provide a suggested diagnosis.

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The method of Claim 1 wherein the charts are selected from Figures 10 to 16.

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The method of Claim 15 wherein in addition the chart is selected from Figures 17 and 18.

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The method of Claim 6 wherein the data bases are selected from Figures 2 to 9.

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A method for diagnosing blood clotting disorders in humans which comprises:

(a)   separately testing samples of plasma from a patient and pooled plasma from normal healthy humans (PNP)

5   for the time to coagulate by prothrombin (PT), activated partial thromboplastin (APT) and thrombin (TCT), charting the results together on a side-by-side basis, and comparing the results from the samples with a data base showing normal ranges of coagulation times (CT) for healthy humans

10 wherein the APT, T and P from separate lots have been prepared in solution to produce a particular standardized clotting time with PNP which is used in all of the testing of the patient plasma;

(b)   optionally testing the samples, of the

15 plasma from a patient and PNP by mixing a blood sample known to be genetically deficient in a blood factor selected from the group consisting of Factors V, VII, VIII, IX, X, XI, XII, Fl.F and HMWK with a volume of a genetic factor deficient plasma GFPP in an amount between about 40

20

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to 60 percent of GFPP to PNP and patient plasma in an
amount between about 40 and 60% by volume patient plasma to
PNP for coagulation by an appropriate one of PT, APT or
both separately, charting the results together on a
side-by-side basis and comparing the results with a second
25   data base showing abnormal and normal ranges of CT based
upon PNP and wherein the CT of GFPD is corrected by the PNP
and normal patient plasma;

        (c) optionally testing the samples of the
plasma for anti-thrombin III by determining CT for the
30   patient and PNP, charting the results together on a
side-by-side basis and comparing the results with an
antithrombin III data base showing normal ranges for PNP;

        (d) optionally testing the samples of the
plasma for protein C by determining CT and charting the
35   results and comparing the results with a protein C data
base showing normal ranges of CT based upon the PNP;

        (e) optionally testing the samples of plasma
for fibrinogen from the patient plasma and PNP and
determining CT for coagulation by T at known dilutions of
40   the plasma with defribrilated PNP, charting the results as
CT on a side-by-side basis and comparing the results with a
fibrinogen data base showing normal ranges of CT for the
PNP; and

        (f) providing a diagnosis based upon the
45   differences in coagulation times in the tests.

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        The method of Claim 18 wherein the data bases
are in a program in a computer.

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        The method of Claim 19 wherein the tests (a) to
(e) are performed.

COAGULATION SCREENING TESTS

ABNORMAL ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)	ABNORMAL PROTHROMBIN TIME (PT)	ABNORMAL THROMBIN CLOTTING TIME (TCT)
DEFICIENCIES: 　FLETCHER FACTOR 　HMW-KININOGEN 　PREKALLIKREIN 　FACTOR XII 　FACTOR XI 　FACTOR IX 　FACTOR VIII	FVII DEFICIENCY	
AFIBRINOGENEMIA FACTOR V DEFICIENCY FACTOR X DEFICIENCY LUPUS ANTICOAGULANT ORAL ANTICOAGULANT LIVER DISEASE POLYCYTHEMIA HEPARIN	AFIBRINOGENEMIA HYPOFIBRINOGENEMIA DYSFIBRINOGENEMIA FACTOR V DEFICIENCY FACTOR X DEFICIENCY LUPUS ANTICOAGULANT ORAL ANTICOAGULANT LIVER DISEASE POLYCYTHEMIA	AFIBRINOGENEMIA HYPOFIBRINOGENEMIA DYSFIBRINOGENEMIA HEPARIN
CLOTTED BLOOD 　(ARTIFACT OR DIC) HEPARIN > 0.4u/ML	CLOTTED BLOOD 　(ARTIFACT OR DIC) HEPARIN > 0.4u/ML	CLOTTED BLOOD 　(ARTIFACT OR DIC) HEPARIN > 0.4u/ML

FIG.1

| SCREENING PROCEDURES AND ASSAYS	IDENTIFIES	TEST RESULTS	PATHOGENESIS	CLINICAL MANIFESTATIONS
PROTHROMBIN TIME ASSAY (PT) NORMAL RANGE: 10.3-12.7 SECONDS	IDENTIFIES: FI, II, V, VII, X SELECTIVE FOR FVII DEFICIENCY SENSITIVE TO PLASMA LEVEL CHANGES: FI, V, VII	LESS THAN 1% DEFICIENCY FV: 48-50 SEC. FVII: 28-30 SEC. FX: 50-52 SEC. FIBRINOGEN; 6MG/DL 32-35 SEC. THERAPUTIC RANGE FOR ANTICOAGULANT THERAPY: 1/1/2 TIMES NORMAL RANGE	NEWBORN INHERITED FACOTR DEFICIENCIES AGED PLASMA WARFARIN LIVER DISEASE LUPUS ANTICOAGULANT POLYCYTHEMIA AFIBRINOGENEMIA	BLEEDING: UMBILICAL CORD, UTERINE, SURGERY, CHILDBIRTH, TRAUMA, ETC.

COAGULATION

FIG. 2-1

| COAGULATION	IDENTIFIES:	LESS THAN 1% DEFICIENCY:		
ACTIVATED PARTIAL THROMBOPLASTIN TIME ASSAY (APTT) NORMAL RANGE: 20.5-30-5 SECONDS	-FACTORS OF THE CONTACT PHASE: PREKALLIKRIEN HMW-K, FXII	HMW-K: 142-158 SEC.		DECREASED FIBRINOLYTIC ACTIVITY
	-FACTORS OF THE INTRINSIC PATHWAY: FXI, IX, VIII	PREKALLIKRIEN 80-90 SEC. FXII: 280-300 SEC. FXI: 138-150 SEC. FIX: 78-82 SEC. FVIII: 77-80 SEC.	INHERITED DEFICIENCIES	INCREASED INCIDENCE OF THROMBOTIC DISEASE
	-FACTORS OF THE COMMON PATHWAY: FI,II,V,X SELECTIVE FOR: FACTORS OF THE CONTACT PHASE AND INTRINSIC PATHWAY	FX: 144-150 SEC. FV: 135-140 SEC.	INHERITED DEFICIENCIES	HEMARTHROSIS AND MUSCLE BLEEDING PSEUDO TUMORS
	SENSITIVITY-CONFERRED BY ACTIVATING AGENT		INHERITED DEFICIENCIES	JOINT AND MUSCLE BLEEDING
	KAOLIN AND SILICA ARE RECOMMENDED ELLAGIC ACID AND SOY EXTRACT ARE UNRELIABLE	HIGH DEGREE OF VARIABILITY WITH HEPARIN THERAPY	LUPUS ANTICOAGULANT HEPARIN THERAPY LIVER DISEASE POLYCYTHEMIA AFIBRINOGENEMIA	

FIG. 2-2

FIG.3
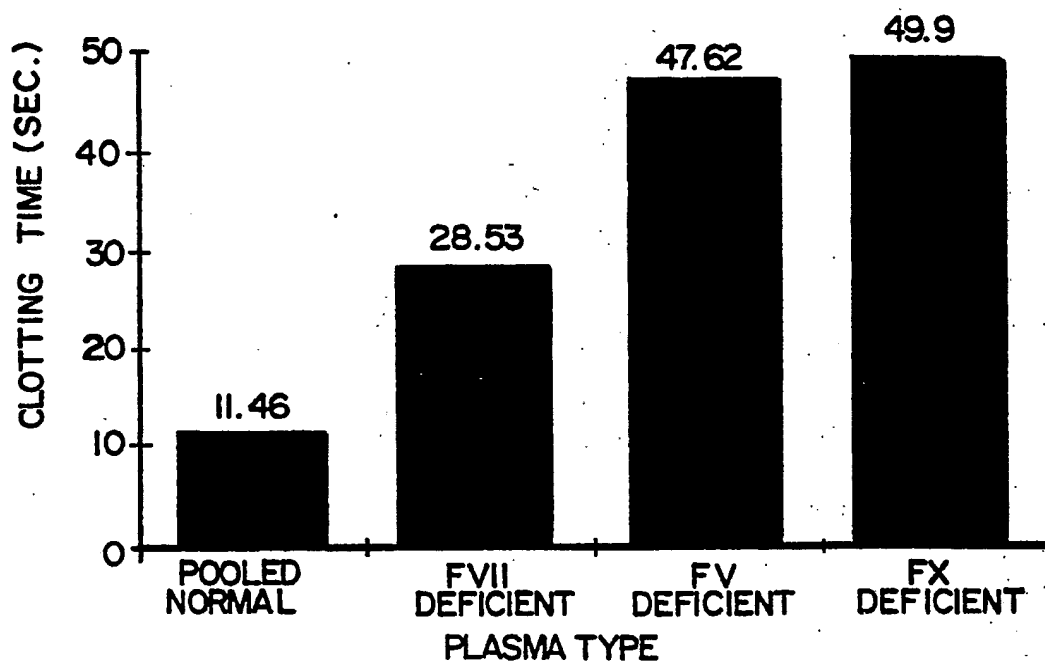
FIG.6
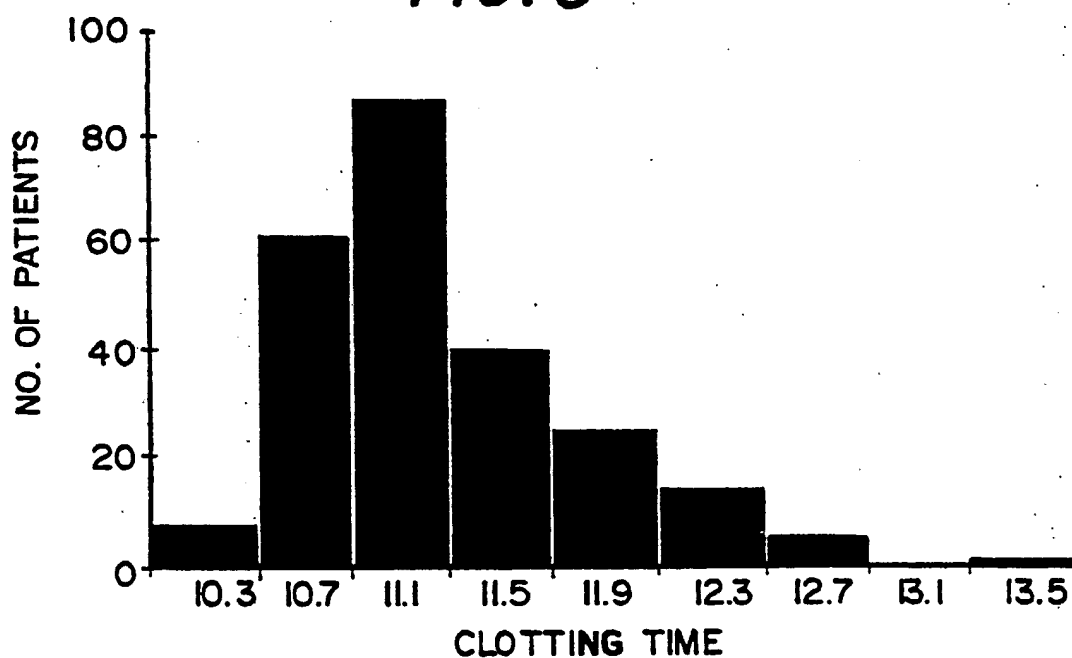
FIG. 4
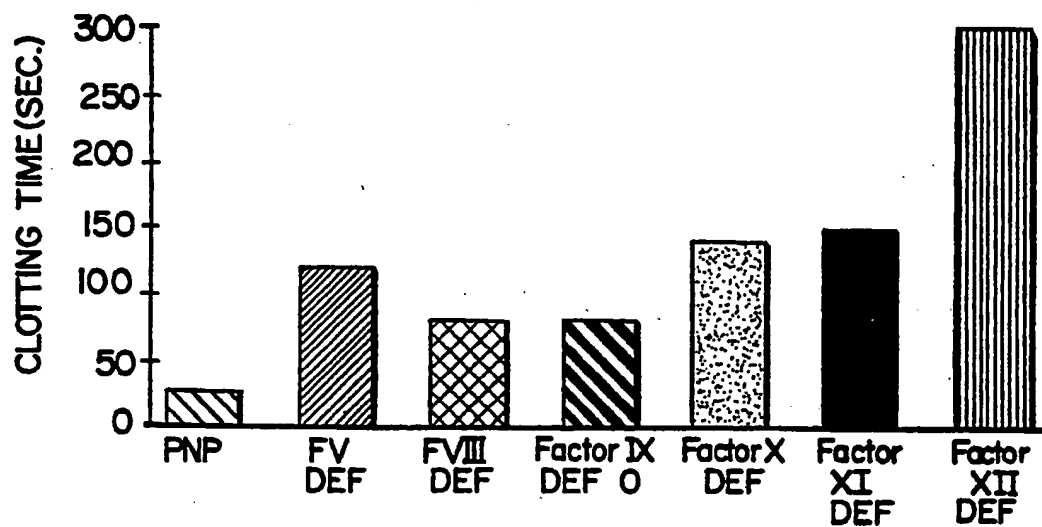
FIG. 7
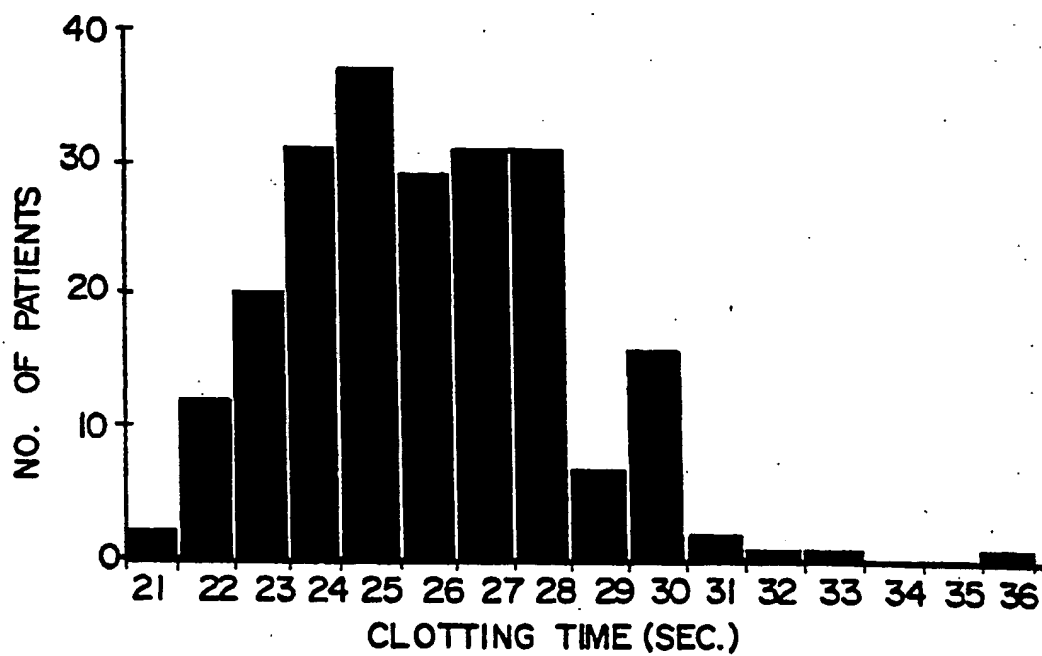
SUBSTITUTE SHEET.

SCREENING TEST RESULTS						POSSIBLE DISORDERS	CONFIRMATORY SPECIFIC ASSAYS
CAPILLARY FRAGILITY TESTS	PLATELET COUNT 150-400 K/MM³	BLEEDING TIME 3-8 MINS.	PT 10.3-12.7 SEC.	APTT 21.5-30.5 SEC.	TCT 7-8 SEC.		
NEGATIVE	150-400 K/MM³	>15 MINS.	NO CLOT	NO CLOT	NO CLOT	AFIBRINO-GENEMIA	CHEMICAL FIBRIN-OGEN THROMBIN CLOTTABLE FIBRINOGEN
NEGATIVE	150-400 K/MM³	3-5 MINS.	11-12.8 SECS.	21-31 SECS.	12-14 SECS.	FETAL FIBRINOGEN LIVER METASTASIS HEPATOMA	CHEMICAL ASSAY THROMBIN CLOTT-ABLE FIBRINOGEN
NEGATIVE	150-400 K/MM³	3-5 MINS.	11-12 SECS.	31-33 SECS.	60 SECS.	HEPARIN	REACT WITH HEPAORB REAGENT QUANTITATIVE HEPARIN ASSAY
NEGATIVE	150-400 K/MM³	3-5 MINS.	10-11 SECS.	240-300 SECS.	7-8 SECS.	FXII DEFICIENCY	FXII ASSAY
NEGATIVE	150-400 K/MM³	8-12 MINS.	48-50 SECS.	135-140 SECS.	7-8 SECS.	FV OR FX DEFICIENCY	FV ASSAY FX ASSAY

FIG. 5-1

Deficiency	Assays						Result
DEFICIENCY OF CONTACT PHASE OR INTRINSIC FACTORS ACQUIRED SPECIFIC INHIBITORS LUPUS-LIKE INHIBITOR	ASSAYS FOR LUPUS LIKE INHIBITOR STUDIES SPECIFIC FACTOR ASSAYS	7-8 SECS.	78-80 SECS.	10-11 SECS.	8-10 MINS.	150-400 K/MM^3	NEGATIVE
ORAL ANTI-COAGULANTS CEPHALO-SPORINS VIT.K DEFICIENCY	FV ASSAY FIX ASSAY	7-8 SECS.	31-35 SECS.	19-23 SECS.	3-8 MINS.	150-400 K/MM^3	NEGATIVE
ADVANCED LIVER DIS-EASE CLOTTED SPECIMEN	FV ASSAY REPEAT PT, APTT, TCT ON FRESH PLASMA SAMPLE	12-14 SECS.	42-45 SECS.	19-23 SECS.	8-10 MINS.	150-400 K/MM^3	NEGATIVE
DYSFIBRINO-GENEMIA DYSPROTEIN-EMIA	CHEMICAL ASSAY THROMBIN CLOTT-ABLE FIBRINOGEN PROTEIN ELECTRO-PHORESIS	16-18 SECS.	21-31 SECS.	14-16 SECS.	8-10 MINS.	150-400 K/MM^3	NEGATIVE
FVII DEFICIENCY	FVII ASSAY	7-8 SECS.	21-31 SECS.	26 SECS.	3-5 MINS.	150-400 K/MM^3	NEGATIVE

FIG. 5-2

		MOLECULAR WEIGHT	PLASMA CONCENTRATION	HALF-LIFE
COAGULATION FACTORS	PROTHROMBIN	72,000	10 MG/DL	48-60 HOURS
	THROMBIN	36,000	——	——
	FACTOR V	300,000	3 MG/DL	12-36 HOURS
	FACTOR VII	45,000-53,000	15-50 MG/DL	5-6.5 HOURS
	FACTOR VIII:C	92,000 AND 80,000 POLYPEPTIDE DOUBLET	0.1-1 MG/DL	2.9 DAYS
	VW FACTOR	POLYMERS 250,000	0.5-1 MG/DL	6-20 HOURS
	FACTOR IX	57,000	0.4 MG/DL	28-40 HOURS
	FACTOR X	54,000	0.8-1 MG/DL	24-56 HOURS
	FACTOR XI	160,000	0.2-0.7 MG/DL	40-80 HOURS
ANTICOAGULANT FACTORS	ATIII	65,000	25 MG/DL	2.5 DAYS
	PROTEIN C	62,000	2.5MG/L+09.MG/L	6-8 HOURS
	HEPARIN COFACTOR II	66,000	6 MG/DL	——
FACTORS OF CONTACT PHASE	HMW-K	120,000	7-10 MG/DL	150 HOURS
	PREKALLIKREIN	85,000	5MG/DL	35 HOURS
	XII	80,000	3-4 MG/DL	50-70 HOURS
FIBRINOLYSIS FACTORS	UROKINASE	55,000	——	20 MIN.
	T-PA	68,000	0.4 MG/DL	2-10 MIN.
	PLASMINOGEN	86,000 SINGLE CHAIN GLU-PLASMINOGEN 80,000 SINGLE CHAIN LYS-PLASMINOGEN	21 MG/DL	2.2 DAYS
	PLASMIN	67,000	0.5 MG/DL	1 MINUTE
FIBRINOLYSIS INHIBIRORS	T-PA INHIBITOR	67,000	0.5 MG/DL	1 MINUTE
	ALPHA-2-PI	50,000	7MG/DL	2.6 DAYS

FIG.8

SUBSTITUTE SHEET

DIAGNOSIS OF ACUTE THROMBOSIS

Clinical Manifestation	Clinical Testing	Radiographic Findings	Laboratory Testing		Identifies
			Serum Enzymes	Hemostasis	
Acute Chest Pain	Inconclusive	Positive Diagnostic	Elevated Diagnostic	Irrelevant	Coronary Artery Thrombosis
Swelling and Edema of Limbs	Inconclusive	Positive Diagnostic	Negative	Antithrombin III↓ Protein C↓ Fibrinogen (no change) Plasminogen↓	Deep Vein Thrombosis
Acute Abdomen	Laparatomy	Positive Diagnostic	Negative	Single Thrombotic Episode: ↓ in Initial Stages of Disease	Mesenteric Thrombosis
Respiratory Distress	Inconclusive	Positive Diagnostic	Elevated	Chronic Lowgrade DIC: ↓ Diagnostic	Pulmonary Embolism
Patches of Hemorrhagic Skin Necrosis Very Ill Patient	Inconclusive	Irrelevant	Elevated	Fibrinogen ↓ Platelets↓ Fibrin Split Products↑ Antithrombin III↓ Diagnostic	DIC

FIG. 9

PATIENT:
SAMPLE FROM:
DATE RECEIVED:
PHYSICIAN:

FACTORS OF THE EXTRINSIC PATHWAY

PATIENT NUMBER:

DATE TESTED:
PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

MIXING STUDIES (PT IN SEC)

	PATIENT(50uL)	CONTROL(50uL)	COMMENTS
GFDP (50UL)			
FACTOR V			
FACTOR VII			
FACTOR X			

REFERENCE RANGE:
CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY INDIVIDUAL DONORS
OF BOTH SEXES, AGES 18 TO 64. NORMAL DISTRIBUTION OF PT, APTT
AND FACTOR LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES OF 223
SINGLE DONOR PLASMA FROM INDIVIDUALS OF BOTH SEXES, AGES 18 TO 64.
GFDP - GENETIC FACTOR DEFICIENT PLASMA

DIAGNOSIS:

FIG. 10

FACTORS OF THE INTRINSIC PATHWAY

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

MIXING STUDIES (APTT IN SEC)

GFDP (50 UL)	PATIENT (50 UL)	CONTROL (50 UL)	COMMENTS
FACTOR V			
FACTOR VIII			
FACTOR IX			
FACTOR X			
FACTOR XI			
FACTOR XII			

REFERENCE RANGE:
    CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                 INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                 NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                 LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                 OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                 BOTH SEXES, AGES 18 TO 64.
                 GFDP = GENETIC FACTOR DEFICIENT PLASMA
    DIAGNOSIS:

FIG. II

INHIBITOR STUDIES

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

PT MIXING STUDIES

GFDP(50uL)	PATIENT(50uL)	CONTROL(50uL)	COMMENTS
FACTOR V			
FACTOR VII			
FACTOR X			
PNP			

APTT MIXING STUDIES

FACTOR V			
FACTOR VIII			
FACTOR IX			
FACTOR X			
FACTOR XI			
FACTOR XII			
PNP			

REFERENCE RANGE:
    CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                  INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                  NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                  LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                  OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                  BOTH SEXES, AGES 18 TO 64.
                  GFDP = GENETIC FACTOR DEFICIENT PLASMA
DIAGNOSIS:

FIG. 12

SUBSTITUTE SHEET

FACTORS OF THE CONTACT PHASE OF PLASMA ACTIVATION

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

APTT MIXING STUDIES

GFDP(50uL)	PATIENT(50uL)	CONTROL(50uL)	COMMENTS
FACTOR XI			
FACTOR XII			
FL.F			
HMWK			

FL.F = FLETCHER FACTOR(PREKALLIKEIN)
HMWK = HIGH MOLECULAR WEIGHT KININOGEN

REFERENCE RANGE:
    CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                    INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                    NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                    LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                    OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                    BOTH SEXES, AGES 18 TO 64.
                    GFDP = GENETIC FACTOR DEFICIENT PLASMA
DIAGNOSIS:

FIG. 13

ANTI-THROMBIN III ASSAYS

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

TCT (DEFIBRINATED PLASMA 100uL)			
TCT + HEPARIN			
TROMBIN INACTIVATED (u/ML)			
% ANITHROMBIN III/HEPARIN COFACTOR ACTIVITY			
IMMUNOREACTIVE LEVELS ATIII			
PROGRESSIVE ACTIVITY ATIII			

NORMAL RANGE = 70 TO 100% PATIENT ATIII ACTIVITY:

REFERENCE RANGE:
       CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                       INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                       NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                       LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                       OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                       BOTH SEXES, AGES 18 TO 64.
                       GFDP = GENETIC FACTOR DEFICIENT PLASMA
DIAGNOSIS:

FIG. 14

PROTEIN C ASSAY

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

PATIENT

ASSAY:	BEFORE ACTIVATION	TTP	SNAKE VENOM	△ ACTIVITY (FOLD DIFFERENCE)
FV CT/SEC.				
FVIII CT/SEC.				
ACTIVITY %				

PERCENT PROTEIN C ACTIVITY:
NORMAL RANGE: 60 TO 100%

PERCENT PROTEIN S ACTIVITY:
NORMAL RANGE: 60 TO 100%

REFERENCE RANGE:
        CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                      INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                      NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                      LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                      OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                      BOTH SEXES, AGES 18 TO 64

DIAGNOSIS:
                      FIG. 15

FIBRINOGEN ASSAY

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

THROMBIN CLOTTING TIME (TCT)	PATIENT		PNP	
PLASMA DILUTIONS	CT/SEC	OGEN MG/DL	CT/SEC	OGEN MG/DL
1 100.00 UL				200.00
2 50.00 UL				100.00
3 25.00 UL				50.00
4 12.50 UL				25.00
5 6.25 UL				12.25
6 3.12				6.25

NORMAL RANGE: 200 TO 400 MG/DL
PATIENT FIBRINOGEN PLASMA LEVEL:

REFERENCE RANGE:
      CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                       INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                       NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                       LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                       OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                       BOTH SEXES, AGES 18 TO 64.
                       GFDP = GENETIC FACTOR DEFICIENT PLASMA
DIAGNOSIS:

FIG. 16

PATIENT REPORT
BLEEDING WORKUP

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

FIBRINOGEN			
FACTOR V			
FACOTR VII			
FACTOR VIII			
FACTOR IX			
FACTOR X			
FACTOR XI			
LUPUS ANTICOAGULANT			
IMMUNOREACTIVE LEVELS OF VWF			
RISTOCETIN COFACTOR ASSAY			

REFERENCE RANGE:
      CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                    INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                    NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                    LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                    OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                    BOTH SEXES, AGES 18 TO 64.
   GFDP = GENETIC FACTOR DEFICIENT PLASMA

DIAGNOSIS:

FIG. 17

SUBSTITUTE SHEET

PATIENT REPORT
HYPERCOAGULABLE WORKUP

PATIENT: PATIENT NUMBER:
SAMPLE FROM:
DATE RECEIVED: DATE TESTED:
PHYSICIAN: PHONE NUMBER TO CALL:

SCREENING TESTS	PATIENT CT/SEC	CONTROL CT/SEC	COMMENTS
PT			
APTT			
TCT			

ANTITHROMBIN III			
PROTEIN C			
PROTEIN S			
PLASMINOGEN			
INHIBITORS TO PA			
FIBRINOGEN			
LUPUS INHIBITOR			
FACTOR XII			
FLETCHER FACTOR			
HMW- KININOGEN			

REFERENCE RANGE:
    CONTROL: POOLED NORMAL PLASMA OBTAINED FROM 40 HEALTHY
                    INDIVIDUAL DONORS OF BOTH SEXES, AGES 18 TO 64.
                    NORMAL DISTRIBUTION OF PT, APTT AND FACTOR
                    LEVELS OBTAINED BY ANALYSIS OF CLOTTING TIMES
                    OF 223 SINGLE DONOR PLASMA FROM INDIVIDUALS OF
                    BOTH SEXES, AGES 18 TO 64.
                    GFDP = GENETIC FACTOR DEFICIENT PLASMA

DIAGNOSIS:

FIG. 18

FIG. 19

FIG. 20

| ◆ PNP   ◇ PATIENT(J.M.)   ■ PATIENT(R.M.)   □ PATIENT(G.D.) |
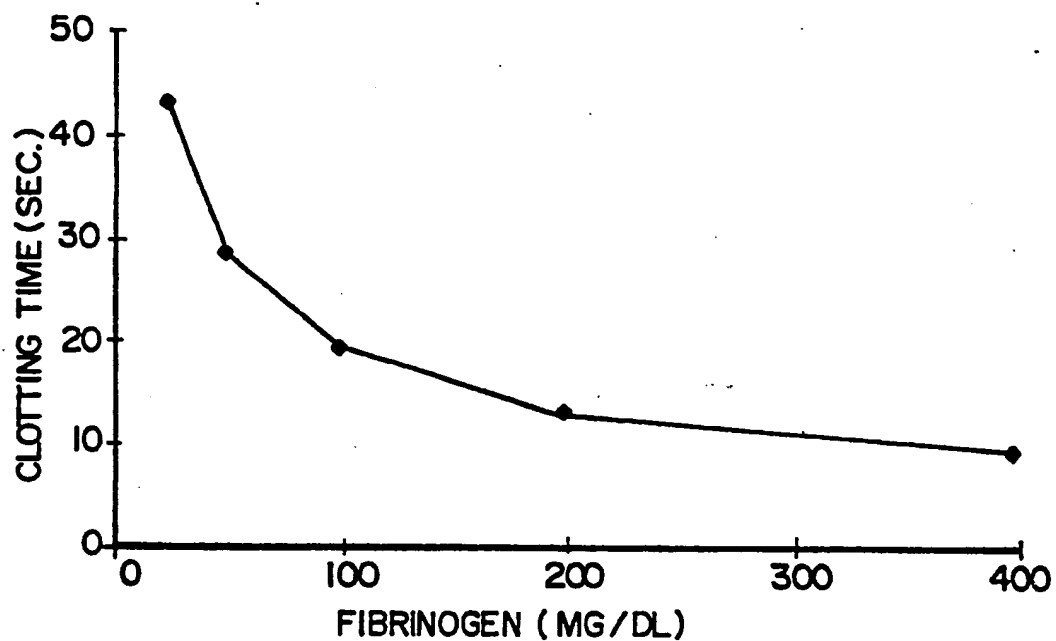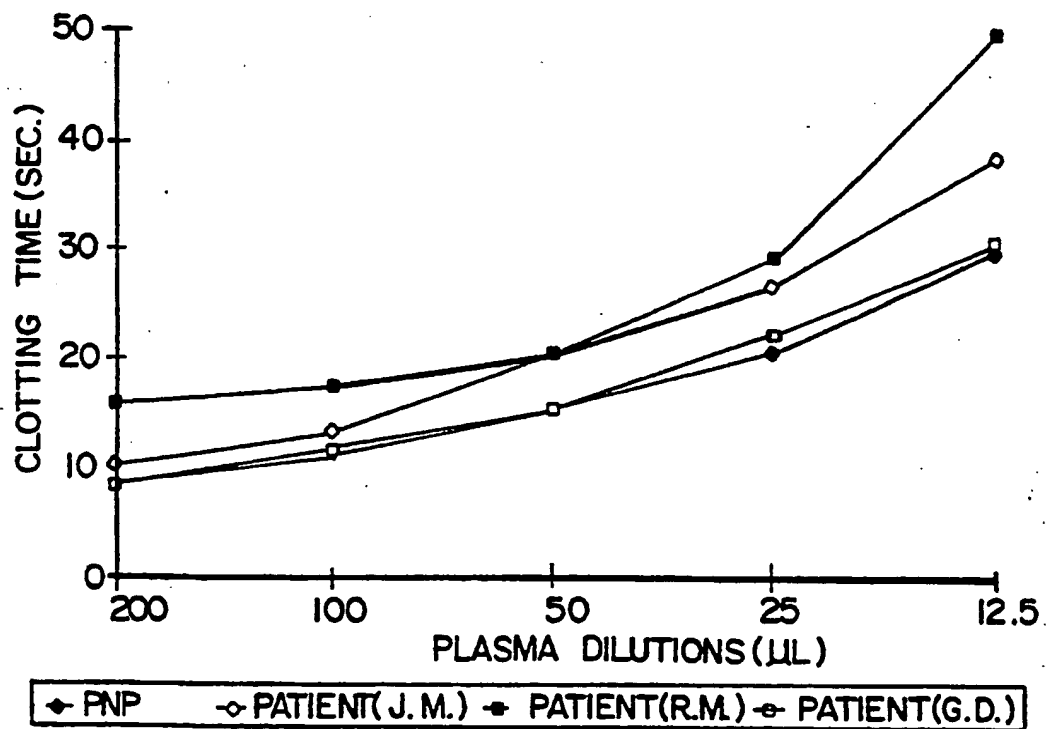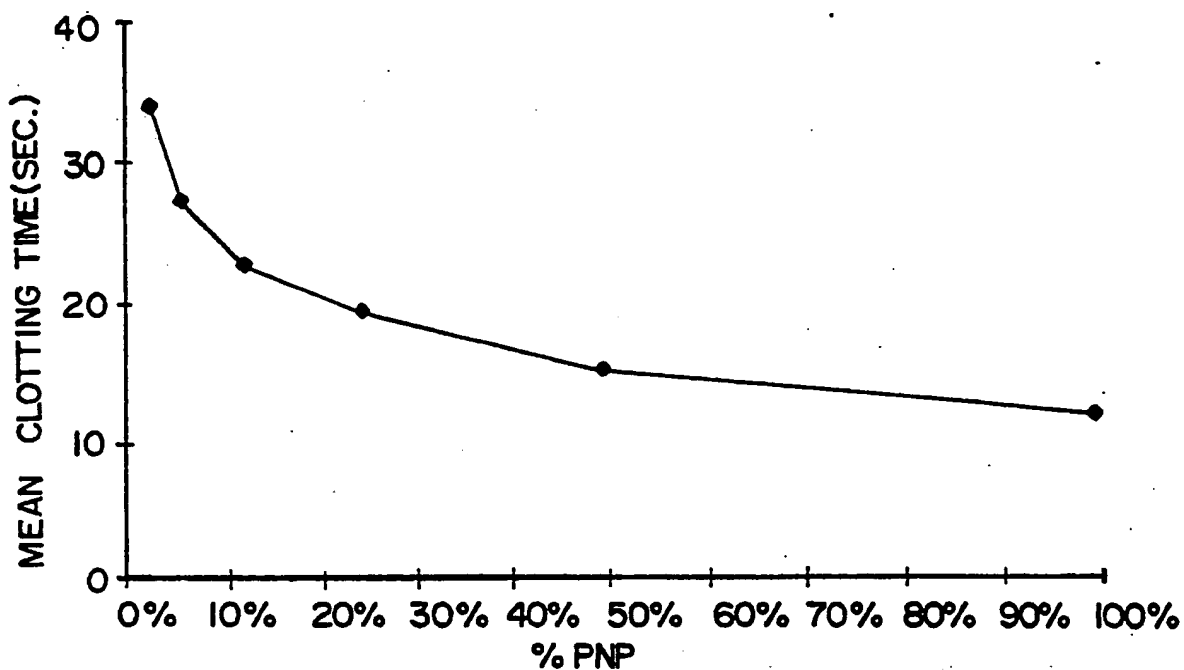
FIG. 21
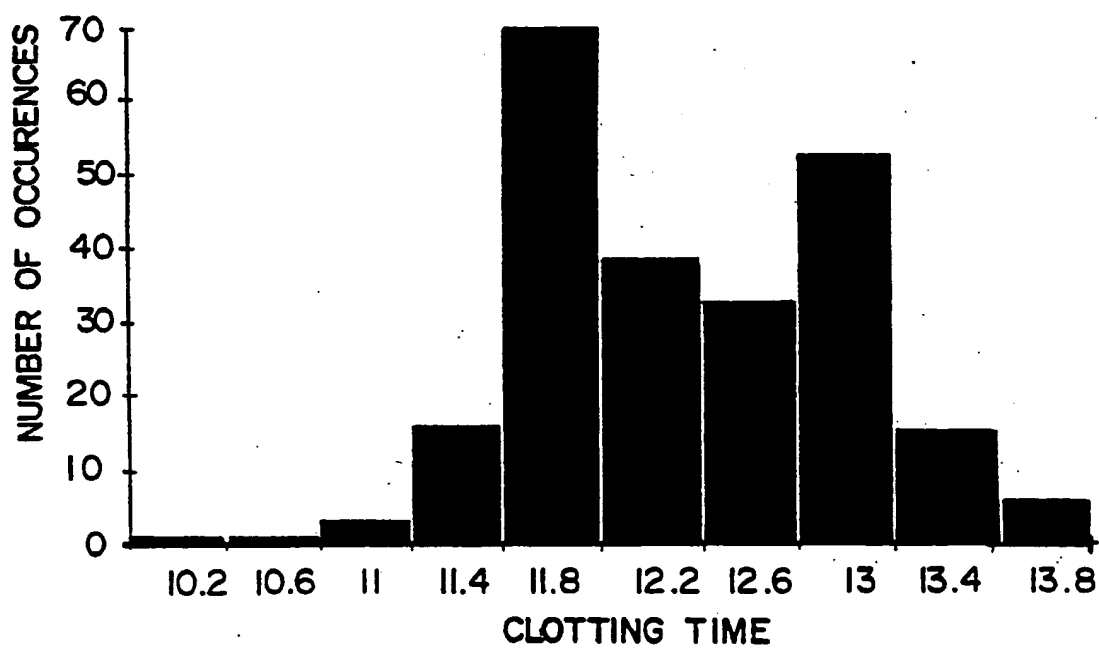
FIG. 24

	THROMBIN ACTIVITY			MEASUREMENT OF PLASMA ATIII/HEPARIN COFACTOR ACTIVITY	
THROMBIN INHIBITED (UNITS)	CLOTTING TIME (SEC.)	THROMBIN MOLES	ATIII MOLES	ATIII µG/100µL	PERCENT ATIII ACTIVITY/100uL
1.5	7.5	1×10^{-11}	1×10^{-11}	0.7	0
1	12	0.7×10^{-11}	0.7×10^{-11}	0.49	30
0.9 TO 0.8	14 TO 16	6.3×10^{-12} 5.6×10^{-12}	6.3×10^{-12} 5.6×10^{-12}	0.42 TO 0.37	40 TO 47
0.7 TO 0.6	18 TO 20	4.9×10^{-12} 4.2×10^{-12}	4.9×10^{-12} 4.2×10^{-12}	0.32 TO 0.25	54 TO 65
0.5 TO 0.4	22 TO 24	3.5×10^{-12} 2.8×10^{-12}	3.5×10^{-12} 2.8×10^{-12}	0.21 TO 0.18	70 TO 74
0.3 TO 0.2	27 TO 28	2.1×10^{-12} 1.4×10^{-12}	2.1×10^{-12} 1.4×10^{-12}	0.14 TO 0.09	80 TO 87
0.1 TO 0.01	30 TO 32	0.7×10^{-12} 0.7×10^{-13}	0.7×10^{-12} 0.7×10^{-13}	0.04 TO 0.004	94 TO 100

FIG. 22
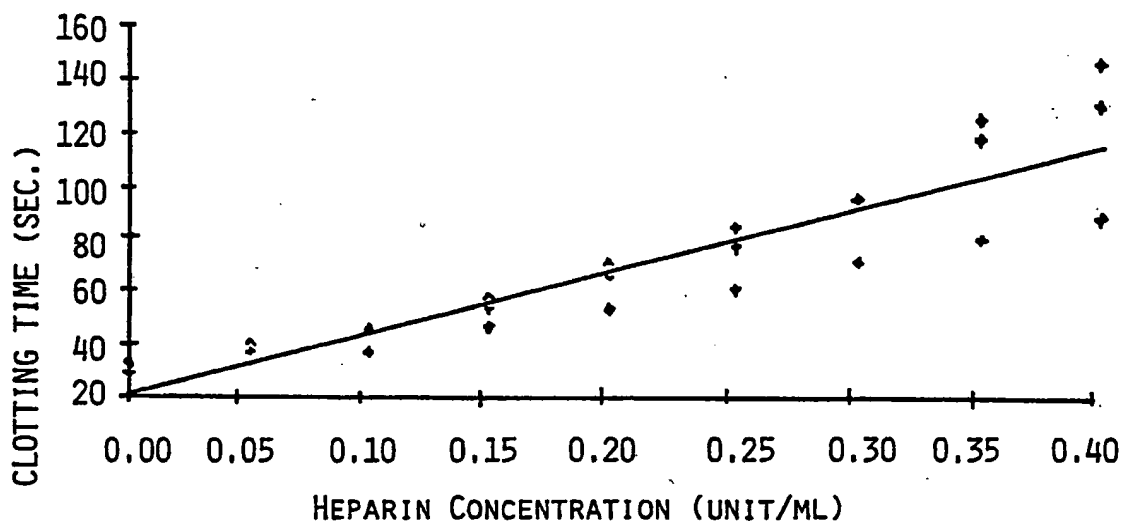
	__A+(B*X)_	__A*EXP(B*X)_	__A+B*LOG(X)_	_A*XcB__
A REG COEFF	21.09037	29.75512	---	---
B REG COEFF	241.9	3.647592	---	---
A STD ERROR	4.895353	1.485747	---	---
B STD ERROR	20.56459	0.2097584	---	---
A T-STAT	4.308243	67.95178	---	---
B T-STAT	11.76293	17.38949	---	---
STD ERR EST	13.79515	0.1407102	---	---
R-SQUARED	0.84697	0.9236395	---	---
COVARIANCE	4.18673	0.0631314	---	---
F-TEST	138.3666	302.3945	---	---
CORR COEFF	0.920309,	0.9610616	---	---
DURBN-WATSN	2.34716	2.954468	---	---

FIG. 23
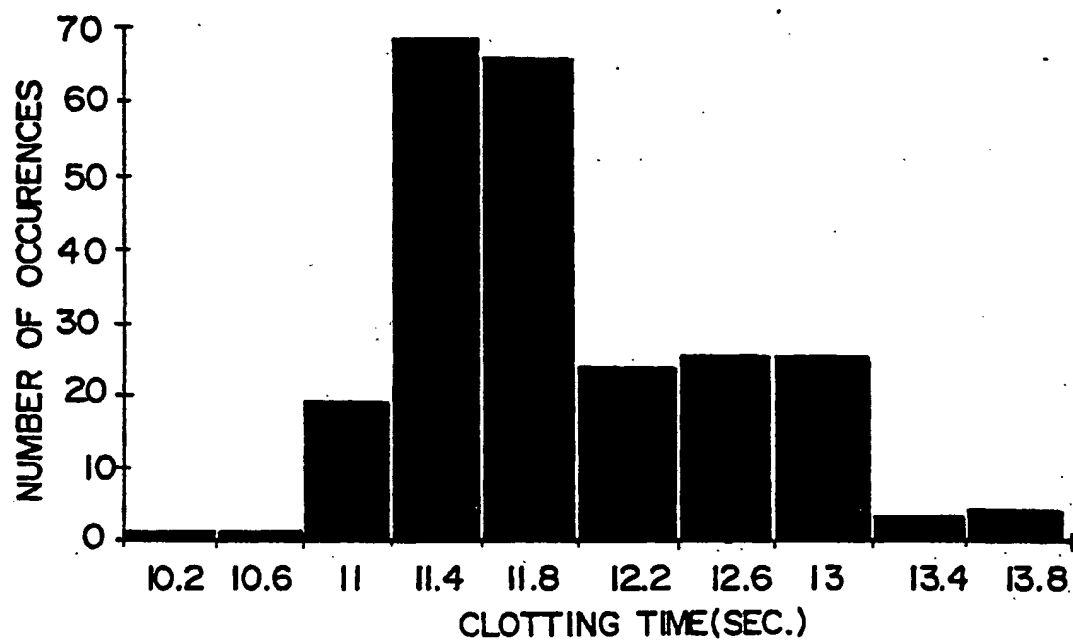
FIG. 25
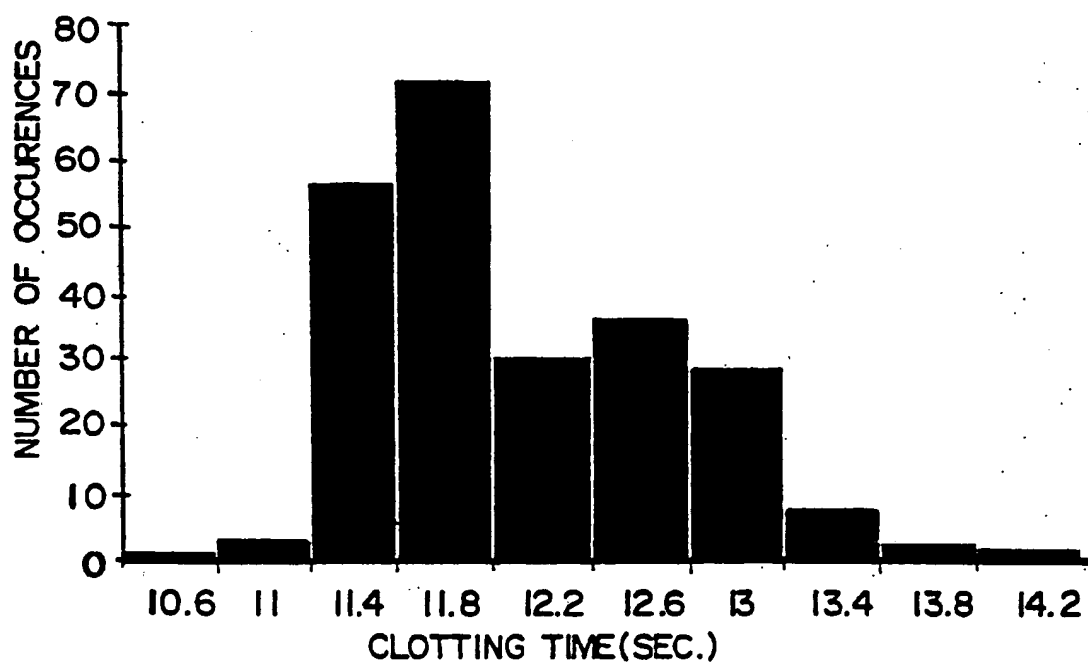
FIG. 26
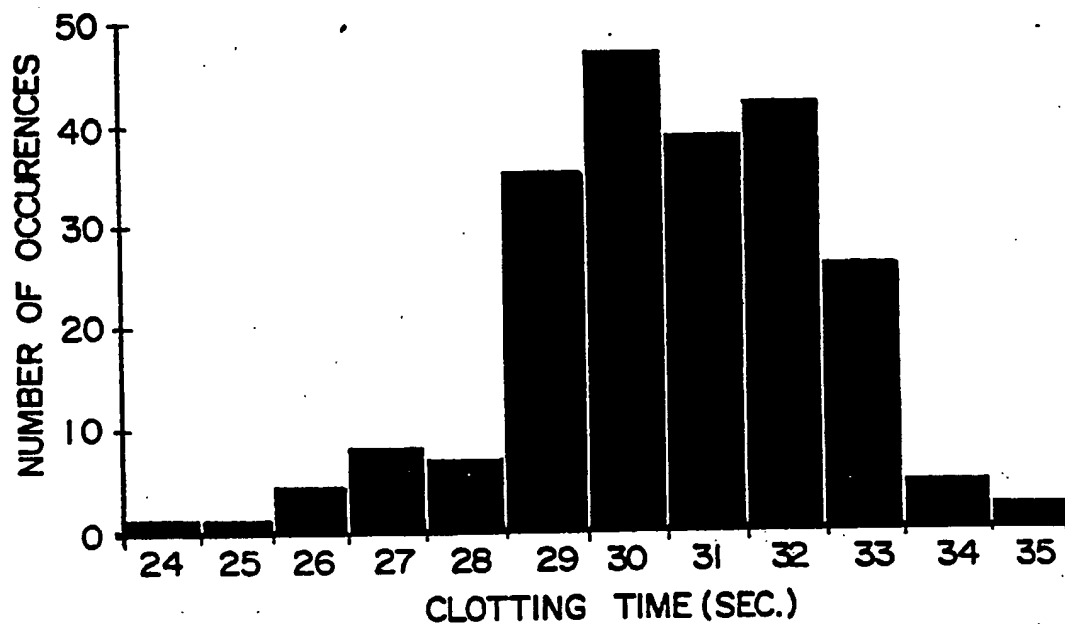
FIG. 27
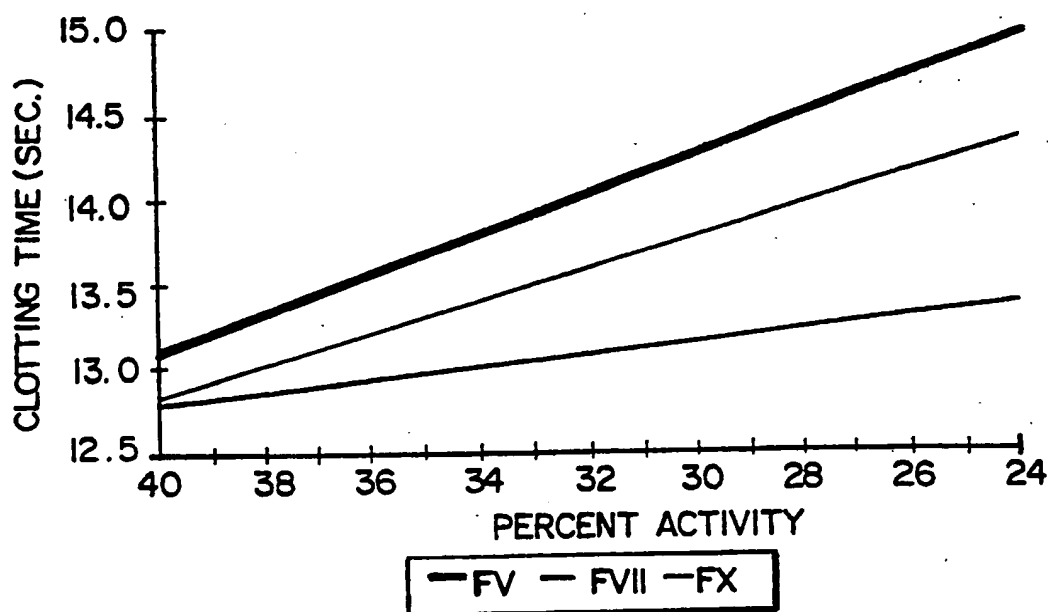
FIG. 28
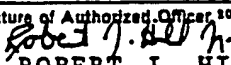
% Factor Activity	FV	FVIII	FIX	FX	FXI
			(Clotting Time in Seconds)		
40	30.38	28.97	30.12	31.53	33.80
35	31.73	30.16	30.61	32.18	34.77
30	33.08	31.35	31.10	32.83	35.73
25	34.43	32.54	31.58	33.48	36.68
20	35.78	33.73	32.06		37.63
16	36.86	34.69			
16	37.1	33.74	33.56	36.34	
12	42.4	37.97	35.93	39.13	
8	47.7	42.20	38.30	41.91	44.59
4	53.0	46.42	40.67	44.70	53.29
2		48.54			57.65
2	65.75	47.54	45.11	47.42	70.95
1.5	77.44	55.61	47.76	52.32	
1	89.14	63.68	50.40	57.23	88.51
0.5	100.84	71.75	53.04	62.13	97.29
0	112.54	79.82	78.54	67.03	

FIG. 29

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/03740

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/56; G01N 33/86
U.S. CL.: 435/13; 436/69

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	436/69; 435/13; 364/497

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	"Gradwohl's Clinical Laboratory Methods and Diagnosis", Volume One Eighth Edition, Edited by Sonnewirth et al, published 1980, by the C.V. Mosby Company, St. Louis, see Chapters 42-45, pp. 1013-1060.	1-20
Y	US, A, 3,486,981 (Speck) 30 December 1969, see especially column 9.	1-20
Y	EP, A, 0,182,929 (American Hospital Supply Corporation) 04 June 1986, see the entire document.	4,6-7, 12-14 and 18-20
Y	"A Closer Look at Hemostasis- An Introduction Coagulation", Ortho Diagnostics, Inc., published 1975, by Ortho Diagnostics, Inc., Raritan, New Jersey, see pages 1-51.	1-20

* Special categories of cited documents: [15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [8]	Date of Mailing of this International Search Report [8]
04 September 1990	02 JAN 1991
International Searching Authority [9]	Signature of Authorized Officer [10]
ISA/US	ROBERT J. HILL, Jr.

Form PCT/ISA/210 (second sheet) (May 1986)